UNIVERSITEIT GENT

FACULTY OF SCIENCES

THE ZEBRAFISH DENTITION IN CONTEXT: EXPLORING THE NEUROVASCULAR ENVIRONMENT

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THE ZEBRAFISH DENTITION IN CONTEXT:

EXPLORING THE NEUROVASCULAR ENVIRONMENT

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AA	aortic arches
A	anterior
Ab	aboral
AMA	anterior mesenteric arteries
BA	bulbus arteriosus
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic protein
br	branch
C	caudal
c.b	ceratobranchials
CNS	central nervous system
CTR	control
CV	caudal vein
D	dorsal
d.p	dental papilla
DA	dorsal aorta
DBS	dental blood sinus
DCC	deleted colorectal carcinoma
DLAV	dorsal longitudinal anastomotic vessel
DMSO	dimethylsulfoxide
dpf	days post fertilization
e.g.	exempli gratia
EC	early cytodifferentiation
Eda	ectodysplasin
edar	Eda receptor
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced GFP
EMT	epithelial to mesenchymal transition
Eph	ephrin receptor
ext	external
Fgf	fibroblast growth factor
fli	friend leukemia integration
flk	fetal liver kinase
flt	Fms-like tyrosine kinase
FT	functional tooth
g	ganglion
GDNF	glial cell derived neurotrofic factor
GFP	green fluorescent Protein
h	hours
HA	hypobranchial artery
HIF	hypoxia inducible factor
hpf	hours post fertilization

hr	hours
i.d.e	inner dental epithelium
i.e.	id est
ins	intestinal
int	internal
IWT	instituut voor innovatie door wetenschap en technologie
JC	Jeroen Crucke
KB	koninklijk besluit
Kdr	kinase insert domain receptor
L	lumen
LC	late cytodifferentiation
LDA	late dorsal aortae
M	morphogenesis
MC	mural cell
MD	mediodorsal
me	mesenchyme
MS	methanesulfonate
MS222	3-aminobenzoic acid ethyl ester
n	number
NGF	nerve growth factor
NRP	neuropilin
o.d.e	outer dental epithelium
obd	out of bounds
Or	oral
p	placode
P	posterior
pax	paired box genes
PBS	phosphate-buffered saline
PBS-T	PBS-tween
PCV	posterior cardinal vein
PDGF	platelet-derived growth factor
PH.C	pharyngeal cavity
ph.e	pharyngeal epithelium
phar	pharyngoclavicularis
pitx	pituitary homebox
PLGF	placental growth factor
prt	pretrematic
pst	posttrematic
R	rostral
RA	retinoic acid
rm	ramus
Robo	roundabout
RT	replacement tooth
SC	sinusoidal cavity
Shh	sonic hedgehog
SL	standard length

sl	successional lamina
SU	SUGEN
Tg	transgenic
TL	total length
TRT	treated
UNC	uncoordinated
V	ventral
VA	ventral aorta
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
vs	vagal sensory
vSMC	vascular smooth muscle cells
Wnt	wingless
WT	wild-type

OUTCOMES RELATED TO THE PHD-PROJECT

1. PEER-REVIEWED PAPERS

- **Crucke, J**. and Huysseune, A. (2013) - Unravelling the blood supply to the zebrafish pharyngeal jaws and teeth. *Journal of Anatomy*, 223, 399-409.

- **Crucke, J**. and Huysseune, A. (2015) – Blocking VEGF signalling delays development of replacement teeth in zebrafish. *Journal of Dental Research*. 94, 157-165

- **Crucke, J.**, Van de Kelft A., Huysseune A. (2015) – The innervation of the zebrafish pharyngeal jaws and teeth. *Journal of Anatomy (In press)*

2. CONFERENCE CONTRIBUTIONS

Crucke J., De Clercq, L., Huysseune, A. (2011) - The vascular supply of zebrafish teeth, first attempts to dissect the neurovascular link. Interdisciplinary Approaches in Fish Skeletal Biology (2011), Tavira, Portugal.

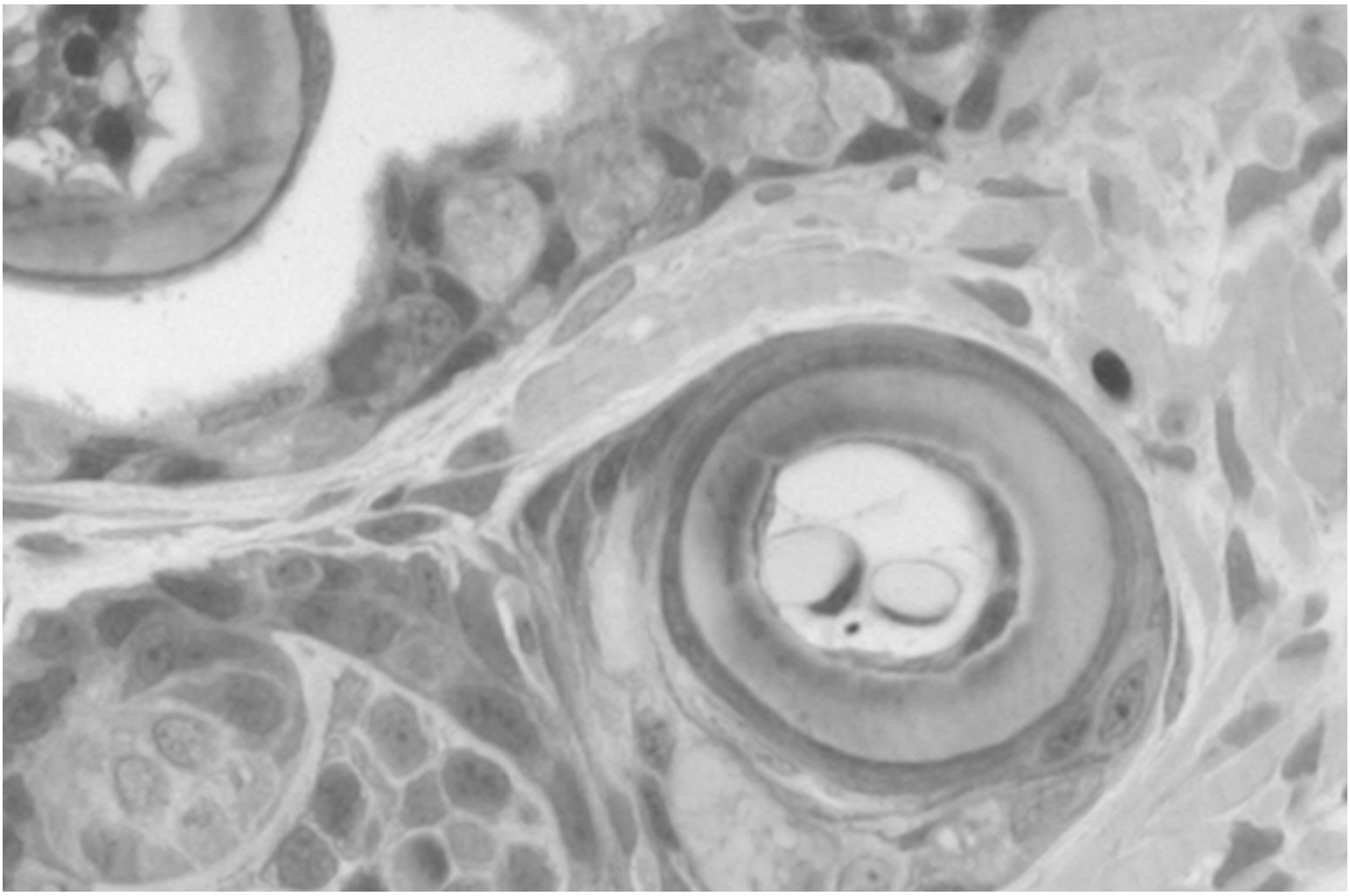
Crucke J., Huysseune A. (2012) - The vascular supply of zebrafish teeth, and its effect on tooth replacement. 17th International Vascular Biology Meeting (2012), Wiesbaden, Germany.

Crucke J., Huysseune A. (2013) - The importance of blood vessels during zebrafish tooth development. 11th International Conference on Tooth Morphogenesis and Differentiation (2013), La Londe, France.

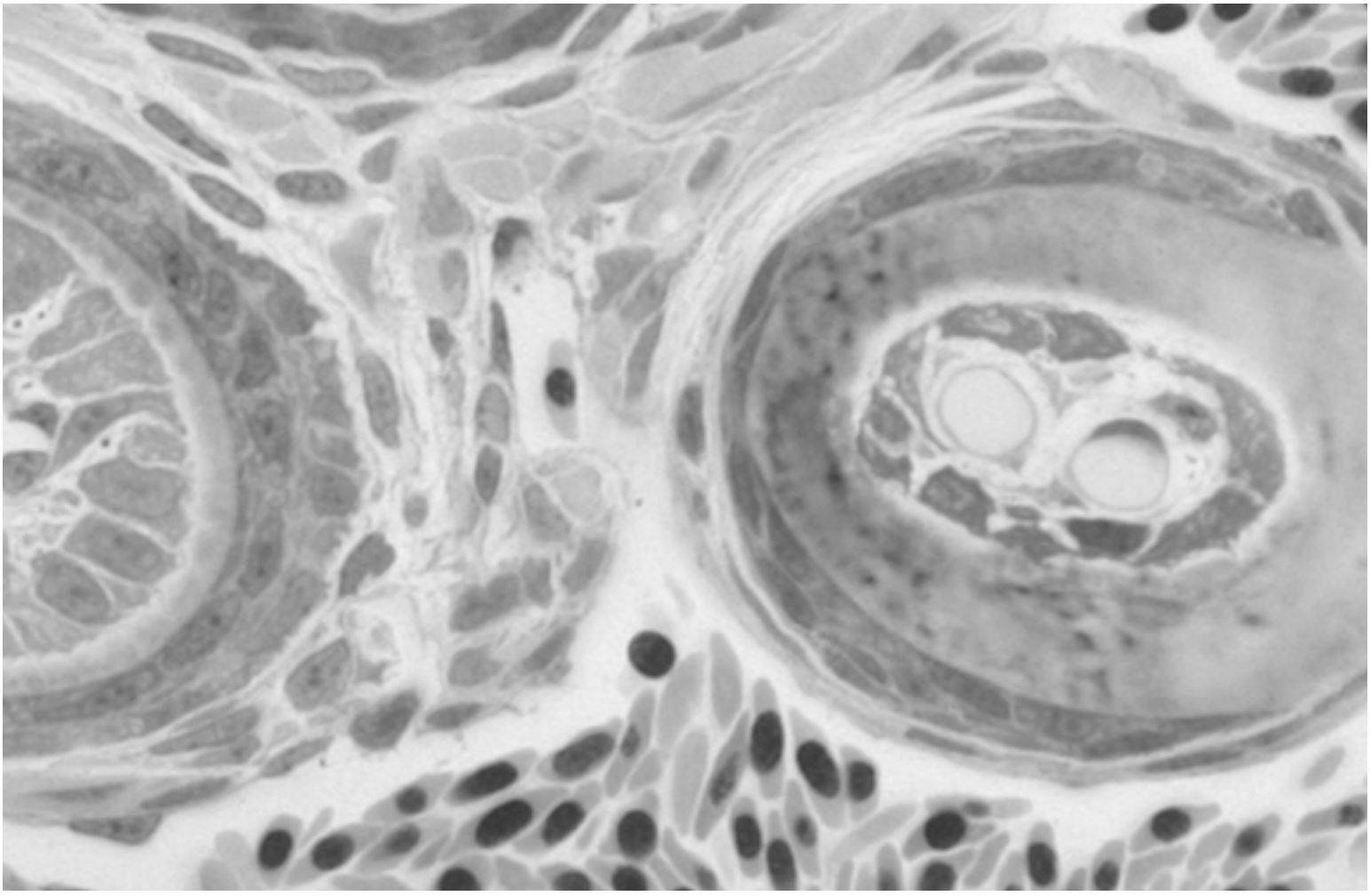
Huysseune A., **Crucke J.** (2015) - Tooth Replacement in an Anatomical Context: Studies in the Zebrafish. The annual Experimental Biology meeting (2015), Boston, MA, United States.*

Crucke J., Huysseune A. (2015) - The zebrafish dentition in context: exploring the neurovascular link. Interdisciplinary Approaches in Fish Skeletal Biology (2015), Tavira, Portugal.*

* not personally attended



INTRODUCTION



1. INTRODUCTION

Throughout scientific history, numerous studies have been devoted to the origin, evolution, organogenesis, and pathology of teeth. These studies have provided information on many levels including the genetic control of tooth development (Jackman et al., 2004, Jarvinen et al., 2006, Thesleff, 2006, Wise and Stock, 2006, Tummers and Thesleff, 2009), and the underlying mechanisms of evolutionary differences in tooth shape, reduction and tooth loss (Jernvall and Thesleff, 2000, Line, 2003, Harada and Ohshima, 2004, Stock et al., 2006). In general, evolution has led to a reduction in the number of teeth (from polyodonty to oligodonty) and of their generations (from polyphyodonty to di- and/or monophyodonty), as well as an increase in morphological complexity of the teeth (from homodonty to heterodonty) (Butler, 1995, Huysseune and Sire, 1998, Salazar-Ciudad and Jernvall, 2004).

Teeth are highly mineralized, usually considered ectodermal appendages that are associated mainly with prehension and processing of food, but also frequently serve other functions, such as defence, display of dominance and production of speech in humans. In general, the main body of a tooth consists of mineralized tissue called dentine, which is produced by odontoblasts, cells of neural crest origin (Lumsden, 1988). Dentine surrounds the pulp that contains nerves and blood vessels in addition to fibroblast-like cells and odontoblasts. Finally, a layer of enamel (mammals) or enameloid (most actinopterygians and all chondrichthyans) covers the outer part of the dentine (Huysseune and Sire, 1998, Van der heyden et al., 2000, Sire et al., 2002). Enamel is produced solely by ameloblasts, whereas enameloid is produced both by odontoblasts and ameloblasts (Wakita, 1993, Sasagawa et al., 2009, Assaraf-Weill et al., 2013, Assaraf-Weill et al., 2014).

Despite the large interest by the scientific community in the field of odontogenesis, certain aspects of tooth development and replacement have remained rather poorly studied. The role of angiogenesis and neurogenesis in tooth development and tooth replacement (and by extension in tooth engineering) is one of them. This is remarkable considering the importance of innervation in organ formation, maturation and homeostasis, and given that vascularization, apart from having an essential nutritive function, also plays a vital role in providing developmental signals to promote organ morphogenesis (Lammert et al., 2001, Thisse and Zon, 2002). In addition, teeth do not function isolated from their environment and require the correct innervation and vascularization in order to perform their lifelong function. The

connection between vascular and neural development, maintenance and functioning is termed the 'neurovascular link' and has received renewed interest over the last few years (Carmeliet, 2003b, Carmeliet and Tessier-Lavigne, 2005, Segura et al., 2009).
In the present work, we have initiated research on the role of the neurovascular link during tooth development and replacement, taking advantage of a vertebrate animal model that is easily amenable to experimentation, and which is characterized by a lifelong natural replacement of teeth: the zebrafish (*Danio rerio*).

1.1 *DANIO RERIO* AS A MODEL SYSTEM FOR STUDYING TOOTH DEVELOPMENT

Studies on tissue interactions regulating tooth development are usually carried out on mammalian teeth. However, these teeth have many derived characteristics and possess several specializations when compared to ancestral teeth. Mammals usually only have one replacement cycle (diphyodonty), a complex tooth shape (heterodonty), and a species-specific number of teeth (Butler, 1995, Huysseune and Sire, 1998). The most commonly used model, the laboratory mouse, possesses a very specialized and derived dentition, even more so than humans. Canines and premolars are lacking, and the dentition per jaw quadrant is limited to non-replacing molars and continuously growing incisors (Peterkova et al., 2006). Zebrafish, *Danio rerio*, on the other hand, offers a number of advantages over mammals. Zebrafish have teeth of a relatively simple shape, that are replaced throughout life (Van der heyden and Huysseune, 2000). All stages of tooth development can be found throughout life, greatly facilitating access to sufficient material to study. In addition, the zebrafish is widely used as a vertebrate model organism for genetic, molecular and developmental research (Lele and Krone, 1996, Roush, 1996) and a wide array of genetic and molecular tools are available. Recently it has also emerged as an attractive model to study processes of angiogenesis (Weinstein, 2002b, Ny et al., 2006) and neurogenesis (Lamora and Voigt, 2009, Friedrich et al., 2010). Finally, the zebrafish pharyngeal dentition has already been proposed as an excellent model to explore the developmental genetic mechanisms of vertebrate dental evolution (Stock, 2007).

1.1.1 TOOTH DEVELOPMENT AND REPLACEMENT IN ZEBRAFISH

Zebrafish are small teleost fish belonging to the cyprinids (Cypriniformes, Cyprinidae). They are indigenous to South Asia, and are broadly distributed across parts of India, Bangladesh, Nepal, Myanmar, and Pakistan where they have been reported to occur in a wide variety of habitat types, including irrigation ditches and rice fields, man-made fish ponds, upper reaches of rivers, and even fast flowing hill streams (Bhat, 2003, Lawrence, 2007, Hussain, 2010). Similar to most other non-mammalian vertebrates, zebrafish replace their teeth throughout life (polyphyodont). However, like other members of the Cypriniformes they lack teeth in the oral cavity, and have their teeth restricted to the fifth ceratobranchials, also termed the pharyngeal jaws (Huysseune et al., 1998, Van der heyden et al., 2000). Zebrafish are generalists, feeding on a wide variety of benthic and planktonic crustaceans, in addition to worms and insect larvae (Spence et al., 2007). Based on what we know from studies conducted in a close relative, the carp (Sibbing, 1979, Sibbing, 1982, Gidmark et al., 2014), we can assume the pharyngeal teeth in zebrafish to serve in the mechanical processing of food particles. This is likely achieved through rhythmic grinding of the teeth against the keratinized pad facing the pharyngeal jaws.

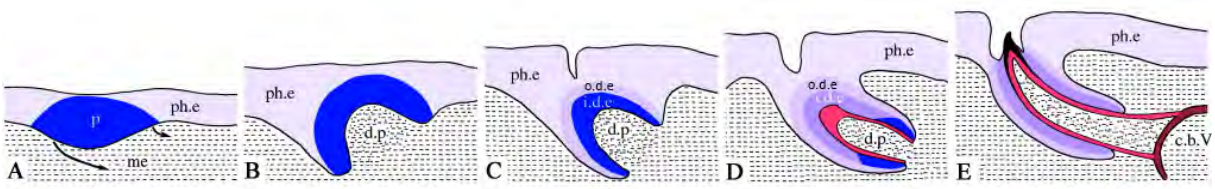


Figure 1: Development of a first-generation tooth
(A) Tooth development starts with a thickening of the pharyngeal epithelium (ph.e), called placode (p), which will further invaginate into the underlying mesenchyme (me). (B-D) This bilayered bell-shaped invagination consist of an inner (i.d.e) and an outer (o.d.e) dental epithelium enclosing a cluster of mesenchymal cells, the dental papilla (d.p). (E) Finally, the tooth becomes attached to the ceratobranchials (c.b) and pierces the pharyngeal epithelium. Stages: (A) initiation; (B) morphogenesis; (C) early cytodifferentiation; (D) late cytodifferentiation; (E) attachment (modified from Laurenti et al., 2004).

Zebrafish tooth development and replacement is a highly regulated process both in time and space (Huysseune, 2006, Huysseune and Witten, 2006). It passes through different, yet partially overlapping stages, starting with initiation, followed by morphogenesis and cytodifferentiation, and ending with attachment of the tooth to the underlying bone. The start of tooth development (initiation stage) can be morphologically observed as a thickening of the pharyngeal epithelium, called placode (Figure 1A), which will further invaginate into a bell-

shaped structure (morphogenesis stage) (Figure 1B-D). This epithelial invagination, or enamel organ, consists of two layers that can be distinguished as an inner and an outer dental epithelium. Enclosed within this bell, is a condensation of mesenchymal cells, the dental papilla, which will further differentiate into odontoblasts and deposit predentine. The cells of the inner dental epithelium on the other hand will differentiate into ameloblasts, and together with the odontoblasts start the production of tooth matrix, enameloid. The differentiation of cells of the dental papilla and of the enamel organ marks the cytodifferentiation stage (Huysseune et al., 1998, Van der heyden and Huysseune, 2000). Finally, attachment to the underlying bone is achieved through a cylinder of attachment bone (Figure 2E). Eruption of the tooth is not accomplished through movement of the tooth itself but rather through a remodelling of the pharyngeal epithelium (Huysseune and Sire, 2004).

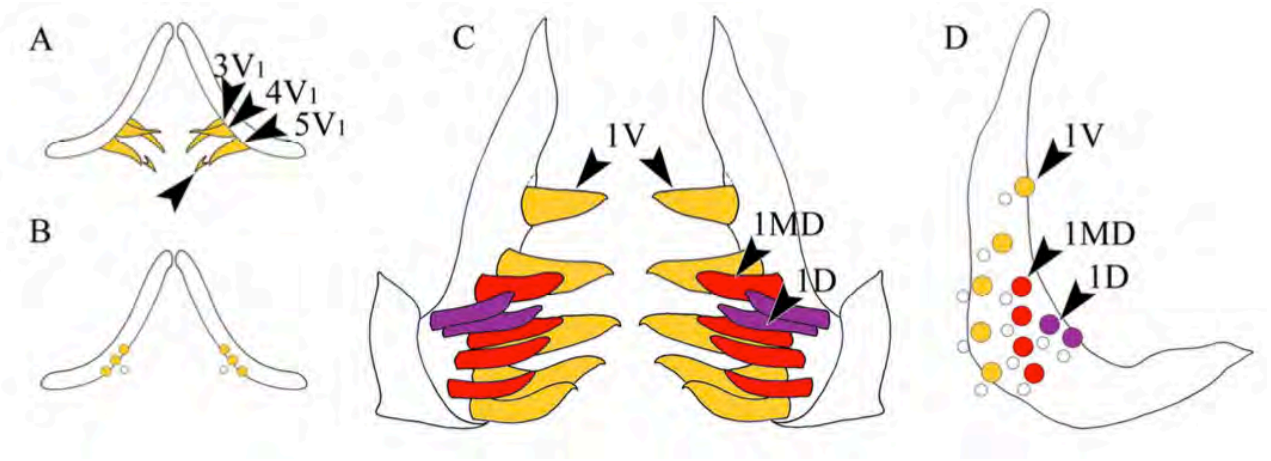


Figure 2: The dentition of zebrafish
(A) The pharyngeal dentition of a larval zebrafish (6 dpf) from a dorsal and slightly posterior view, showing the first three developing teeth ($3V^1$, $4V^1$, $5V^1$) and their position on the fifth ceratobranchials. At this stage, the first replacement tooth $4V^2$ can already be observed (arrowhead). (C) Dorsal view of the complete zebrafish dentition (adult zebrafish) with all tooth positions occupied. Note the organization of the dentition in a ventral row (yellow) with five teeth, a mediodorsal row (red) with four teeth, and a dorsal row (purple) with two teeth. (B, D) Schematized drawing of dentition shown in A and C, respectively from a dorsal (B) and medial (D) view. The small grey circles in (B and D) represent the replacement teeth, each of which is associated with a functional tooth (large coloured circles) (reprinted from Huysseune et al., 2005).

The adult zebrafish dentition consists of three tooth rows that extend rostro-caudally. The ventral (V) row has five teeth, the mediodorsal (MD) row has four, and the dorsal (D) row bears two teeth (Figure 2). The spatiotemporal pattern of tooth development and replacement

in zebrafish has been well characterized in earlier research (Van der heyden and Huysseune, 2000). In short, at 48 hours post-fertilization (hpf), the development of the first tooth at position $4V^1$ is initiated. Shortly after (at 56 hpf), the two neighbouring teeth on the same row (tooth $3V^1$ and $5V^1$) start to develop at approximately the same time and continue to develop near-simultaneously. At that time, tooth $4V^1$ is already in early cytodifferentiation stage. At 80 hpf tooth $4V^1$ has become functional and the replacement tooth $4V^2$ (the second-generation tooth at the fourth position on the ventral row) is initiated. At six days post-fertilization (dpf), tooth $3V^1$ and $5V^1$ are attached and their replacement tooth has started to form (Figure 2A, B). The two remaining teeth on the ventral row, tooth $2V^1$ and $1V^1$, appear at 12 dpf and 16 dpf, respectively. Prior to the development of the last tooth on the ventral row, the first tooth on the mediodorsal row already starts to develop (tooth $3MD^1$). At 20 dpf, the two adjoining teeth on the mediodorsal row develop, followed by the onset of tooth formation in the last tooth position on the mediodorsal row together with the development of the first tooth of the dorsal row. At four weeks post-fertilization all 11 tooth positions are occupied (Figure 2 C, D) (Van der heyden and Huysseune, 2000).

Contrary to first-generation teeth, replacement teeth do not arise directly from the pharyngeal epithelium, but rather from an epithelial invagination that develops from the crypt associated with the erupted predecessor tooth (Huysseune and Thesleff, 2004, Huysseune, 2006). This epithelial downgrowth is termed a successional lamina and is found in association with every functional tooth in the dentition. The distal end of this structure develops into a replacement tooth. The successional lamina itself is quickly taken up into the enamel organ of the developing tooth bud (Huysseune, 2006). It has been proposed that the crypt epithelium from which the new tooth germ buds off contains stem cells responsible for the continuous renewal of teeth in zebrafish (Huysseune and Thesleff, 2004).

The replacement of teeth already starts before all tooth positions are occupied, and is initiated during successive waves, called odontogenic waves. This can be defined as a temporal event in which new tooth germs are initiated simultaneously, which results in a set of teeth of the same age. Successive teeth can be considered as constituting a tooth family (Van der heyden and Huysseune, 2000).

1.1.2 SIGNALLING PATHWAYS REGULATING TOOTH DEVELOPMENT IN ZEBRAFISH

Mammalian tooth development is a highly regulated process involving ligands, receptors, and downstream effectors from multiple pathways. It is the results of reciprocal interactions

between the dental epithelium and the underlying mesenchymal cells. These epithelial-mesenchymal interactions involve signalling pathways such as Bmp (bone morphogenetic protein), Fgf (fibroblast growth factor), Wnt (Wingless), Shh (Sonic Hedgehog), and Eda (ectodysplasin) pathways that are used reiteratively during advancing tooth development. Modification of one of these components through deletion or overexpression usually results in a tooth phenotype (Thesleff and Sharpe, 1997, Thesleff, 2006).

In mice, the dental placode is considered the first signalling centre during odontogenesis. The epithelium induces morphogenesis in the underlying mesenchyme through the secretion of Fgfs and Bmps. In its turn, the mesenchyme responds by sending reciprocal signalling molecules, such as Fgfs and Bmps (Thesleff and Sharpe, 1997, Thesleff, 2003). Blocking Fgf signalling has demonstrated a developmental arrest during early stages of tooth development in both molars and incisors (Mandler and Neubuser, 2001). In addition, Fgf8 has been shown to regulate the expression of Dlx2, Pax9, and Pitx2 during murine tooth development (Kettunen and Thesleff, 1998, Abu-Issa et al., 2002). In zebrafish, Fgf signalling has been demonstrated to be required during early steps of odontogenesis. Moreover, blocking Fgf signalling eliminates all odontogenic gene expression except for *Pitx2*. Knocking down *Fgf4* or *Fgf3* through morpholino injection only results in a mild effect on tooth development (Jackman et al., 2004, Jackman et al., 2013).

Shh signalling is essential during murine tooth initiation and subsequent morphogenesis and differentiation of the dental epithelium into ameloblasts (Hardcastle et al., 1998, Gritli-Linde et al., 2002). Its expression follows that of *Fgf8* and *Fgf9*, suggesting that shh signalling may be downstream of Fgf (Thesleff and Sharpe, 1997). In zebrafish, the hedgehog signalling pathway is shown to be essential for proper tooth initiation and further development (Jackman et al., 2010).

The importance of the Wnt signalling pathway during murine tooth development has already been suggested several times (Jarvinen et al., 2006, Chen et al., 2009, Wang and Fan, 2011). *Lef1* knockout (KO) mice lack teeth as their development is arrested in bud stage (Vangenderen et al., 1994), whereas overexpression of *Lef1* in epithelial cells of transgenic mice results in an increased invagination of the epithelium and the formation of extra tooth like structures (Zhou et al., 1995). In other cycling structures such as hairs, Wnt signalling also plays an important role (Andl et al., 2002). In zebrafish, the masterblind mutant, which contains a point mutation in *axin1* and thus mimics Wnt overexpression, has an overall dramatic phenotype but teeth develop and are replaced in a normal way (van de Water et al., 2001, Huysseune et al., 2014).

Finally, studies on tooth development in mouse have highlighted the importance of the Edar/Eda interactions in regulating enamel knot formation during tooth morphogenesis (Tucker et al., 2000). In zebrafish, recent studies have succeeded in restoring teeth to the upper pharynx through overexpression of the Eda gene. These results indicate that regional loss of Eda expression could have played an evolutionary role in the reduction of the dentition (Aigler et al., 2014).

1.1.3 A ROLE FOR STEM CELLS DURING TOOTH DEVELOPMENT AND REPLACEMENT?

Most non-mammalians replace their teeth throughout life. In zebrafish, at regular time intervals, an epithelial thickening develops from the epithelial fold that surrounds the exposed tip of the functional tooth, giving rise to a new tooth. Huysseune and Thesleff (2004) hypothesized that in order to maintain the continuous replacing of teeth, epithelial stem cells might be located in the epithelium from which the new tooth germ buds off (Huysseune and Thesleff, 2004). In addition, recent studies have identified both periendothelial cells (pericytes) and peripheral nerve-associated glia as a possible source of mesenchymal stem cells (Crisan et al., 2012, Kaukua et al., 2014), which is important to take into account when studying the connection between tooth development and the neurovascular environment. Finally, in mice, epithelial stem cells have already been identified in the cervical loop of the continuously growing incisor, whose descendants contribute to the ameloblast population (Harada et al., 1999). Hence, a possible role for stem cells during continuous tooth development and replacement seems plausible. Therefore, ongoing research is trying to identify potential stem cell niches in both actinopterygians and sarcopterygians, and unravel the role of stem cells in the process of continuous tooth replacement (Harada and Ohshima, 2004, Buchtova et al., 2008, Handrigan et al., 2010, Vandenplas et al., 2014).

In general, a stem cell can be defined as an undifferentiated cell that, upon division, produces one cell that remains undifferentiated, becoming the next stem cell, and one cell that undergoes proliferation, and can differentiate in nearly all tissues within the organism (Fuchs and Segre, 2000). In mammals, there are two broad types of stem cells: embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in various tissues (Seaberg and van der Kooy, 2003). Adult stem cells are mainly involved in the maintenance and repair of damaged organs and tissues and occur in certain stem cell niches, highly regulated microenvironments that maintain the stem cells and their function and prevent them from dedifferentiation (Li and Xie, 2005). In addition, an important

distinction has to be made between embryonic stem cells and adult stem cells in terms of potency. Whereas embryonic stem cells are pluripotent or totipotent and can form nearly all tissues within the embryo, adult stem cells have already been developmentally committed and are multipotent or unipotent (Blanpain et al., 2004). Progenitor cells on the other hand, also have the capacity to differentiate into a specific type cell, but contrary to adult stem cell do not have the ability to replicate indefinitely and can only divide a limited number of times (Seaberg and van der Kooy, 2003).

1.2 THE CARDIOVASCULAR SYSTEM OF *DANIO RERIO*

In vertebrates, the first organ system to become functional is the cardiovascular system. It develops when the need for oxygen and nutrition can no longer be met by means of diffusion alone, because of an increase in size or metabolic rate of the organism (Burggren and Pinder, 1991, Nikolova and Lammert, 2003). Hence, further survival of the embryo depends on the development of the vascular system. In general, the closed circulatory system of vertebrates is composed of a hierarchical network of tubes, consisting of arteries, veins, and capillaries (Hartenstein and Mandal, 2006). Lining the walls of the vascular tubes are endothelial cells (ECs) resting on a basal membrane that recruit supportive mural cells to the tube periphery. These mural cells, vascular smooth muscle cells (vSMCs) or pericytes, regulate endothelial cell function and stabilize vessel integrity (Armulik et al., 2005). Blood vessels are initially formed through vasculogenesis, the *de novo* formation of vessels from precursor cells. Subsequent vascular formation occurs via so called angiogenesis, whereby endothelial tip cells migrate along an extracellular gradient of vascular endothelial growth factor (VEGF), especially during the initiation of angiogenesis (Carmeliet, 2000). Below we will discuss the general characteristics of the cardiovascular system in zebrafish.

1.2.1 MAIN PARTS OF THE CARDIOVASCULAR SYSTEM

In zebrafish, the cardiovascular system is a simple loop with the heart, gills, and systemic circulation in series. Blood vessels are anatomically divided into arteries, capillaries, and veins. Arteries mainly function as compliance vessels and serve in dampening the ventricular pulse, hence protecting the capillaries from excessive pressure. Capillaries are sites for

exchange between blood and tissues. Finally, veins are responsible for returning blood to the heart (Olson, 2000a, Olson, 2000b).

The heart

The heart consists of four chambers: sinus venosus, atrium, ventricle, and bulbus arteriosus. Deoxygenated blood is pumped via the sinus venosus into the atrium and subsequently into the ventricle, then passes through the bulbus arteriosus in the ventral aorta. Oxygenation occurs at the gills after which blood is distributed throughout the body (Hu et al., 2000, Hu et al., 2001). The sinus venosus is a thin-walled reservoir the wall of which consists of connective tissue and some cardiac muscle enabling a weak, but probably insignificant contraction (Santer, 1985). The following chamber, the atrium, has slightly thicker walls than the sinus. The ventricle, on the other hand, has a much thicker wall than the atrium. The wall contains a compact outer layer of muscle, and a spongy inner layer with numerous trabeculae. Finally, the bulbus arteriosus can already be considered as part of the arterial system and has a three-layered wall similar to the larger arteries (see below) (Hu et al., 2000).

Arteries

The arterial vessel wall has three layers (from inside to outward): the tunica intima, tunica media, and tunica adventitia. The relative thickness of each layer varies with the vessel's function. The tunica intima is lined on the luminal side by a single layer of endothelial cells and on the abluminal side by a thin, fenestrated elastic sheet, the internal elastic lamina. The tunica media consists mainly of smooth muscle cells. In some vessels dense elastic fibres are condensed into a sheet forming an outer elastic lamina. The tunica adventitia is commonly a thin layer of collagen with some elastin. The smallest and narrowest of arteries are termed arterioles, with their lumen sometimes as small as a single red blood cell. Their wall consists of a thick layer of smooth muscle cells and a thin layer of elastic tissue (Satchell, 1991, Olson, 2000a, Olson, 2000b).

Capillaries

Capillaries are the smallest of all the blood vessels and make up the microcirculation of the body. They are responsible for the exchange of oxygen and nutrients between blood and tissues. The capillary wall consists of a single layer of endothelial cells surrounded by a basement membrane. These vessels are typically 4-10 µm in diameter and 500-1000 µm long. There are three main types of capillaries: continuous, fenestrated, and sinusoidal. Continuous

capillaries possess a wall where the endothelial cells provide an uninterrupted lining, only allowing the passage of smaller molecules such as water and ions. Fenestrated capillaries have pores in their walls (60-80 nm in diameter) allowing the passage of small molecules and some protein. Finally, sinusoidal capillaries are a special type of fenestrated capillaries also termed discontinuous capillaries. In combination with a discontinuous basal lamina these vessels allow the passage of even red and white blood cells (Satchell, 1991, Olson, 2000a, Olson, 2000b).

Veins

Veins and venules possess a similar three-layered wall as arteries, although they have proportionally thinner walls and less smooth muscle (Satchell, 1991).

1.2.2 THE MAIN CELL TYPES LINING BLOOD VESSELS

The blood vessel wall is composed of two main types of cells. Endothelial cells which line the wall of the different vessels, and vascular mural cells which mainly fulfil a supporting function for the vasculature. Below we will briefly discuss both cell types.

Endothelial cells

Nearly all tissues depend on the presence of a blood supply, and the blood supply in its turn depends on endothelial cells, which form the lining of blood vessels. Although the amount of muscle and connective tissue may vary greatly according to a vessel's size and function, the endothelial lining is always present. Thus, endothelial cells line the entire vascular system, from the heart to the smallest capillary, and control the passage of materials into and out of the bloodstream (Satchell, 1991). Endothelial cells have the capacity to adjust their number and arrangement, through processes of angiogenesis, in response to cues (e.g. HIF-1) from local environments. This capacity ensures that all tissues within the organisms are sufficiently supplied with oxygen and nutrients (Semenza, 2000). In addition, endothelial cells have also been shown to respond to hemodynamic forces, which results from the circulatory flow inside the vessel. Several studies have shown that endothelial cells not only have the capacity to sense hemodynamic forces, but also to distinguish between different types of biomechanical stimuli (Chen et al., 2001, Garcia-Cardena et al., 2001, McCormick et al., 2001, Brooks et al., 2002, Wasserman et al., 2002).

Vascular mural cells

Apart from endothelial cells, the development and maturation of the cardiovascular system requires the recruitment of smooth muscle cells in a process called vascular myogenesis. Smooth muscle cell precursors, called mural cells, will differentiate and surround blood vessels (Carmeliet, 2000, Jain, 2003, Semenza, 2007). During development, these vascular mural cells undergo proliferation and/or differentiation depending on the size of the vessels. On large vessels, they become multilayered and are referred to as vascular smooth muscle cells. On smaller vessels, mural cells remain as single solitary cells embedded within the basement membrane surrounding the endothelial cells, and are usually referred to as pericytes (Santoro et al., 2009). Larger vessels possess dense populations of vascular smooth muscle cells, serving in withstanding higher blood pressures and regulating blood flow. Smaller vessels are loosely covered with pericytes, which serve in regulating vasoconstriction and vasodilation (Wang et al., 2014). Recently, pericytes have received increasing interest from the field of regenerative medicine with respect to their multipotential differentiation capacity (Crisan et al., 2008, Cantoni et al., 2015).

Several factors have been shown to influence mural cell development and differentiation, including endothelial, neuronal and hemodynamic influences (Owens, 1995). It is reasonable to assume that endothelial cells play a crucial role in the development and differentiation of vascular mural cells, since mural cells are recruited to surround the endothelium during development. Indeed, endothelial cells have been shown to secrete growth factors that either inhibit (e.g. heparin) or stimulate (e.g. PDGF) the growth of cultured smooth muscle cells (Owens et al., 1988, Stouffer and Owens, 1994). In addition, the nervous system has also been implicated in the regulation of growth and differentiation of mural cells, since interruption of neuronal input has led to decreased growth and loss of contractility in various smooth muscle tissues of the rabbit ear (Bevan and Tsuru, 1981). Finally, evidence exists indicating an influence of mechanical factors or hemodynamic forces on the development of vascular mural cells. In avian embryos, incorporation of smooth muscle cells coincides with the establishment of blood flow and an increase in luminal hydrostatic pressure (Wagman et al., 1990), which matches with an increased expression of smooth muscle cell differentiation markers at this developmental stage (Hu and Clark, 1989).

Studying the development and distribution of mural cells in zebrafish is usually accomplished through transmission electron microscopy (Figure 3) or immunohistochemical detection of alpha smooth muscle actin, one of the earliest markers of vascular mural cell development that is expressed by both vascular smooth muscle cells and pericytes (Santoro et al., 2009, Wang et al., 2014). In addition, the first transgenic lines marking vascular mural cells are available (Whitesell et al., 2014). This has resulted in the identification of vascular mural cells in zebrafish around the lateral dorsal aortae (LDA), anterior mesenteric arteries (AMA), and the brain of developing animals. Moreover, they have also been demonstrated to contribute to the outflow tract of the developing heart and ventral aorta (Santoro et al., 2009, Wang et al., 2014).

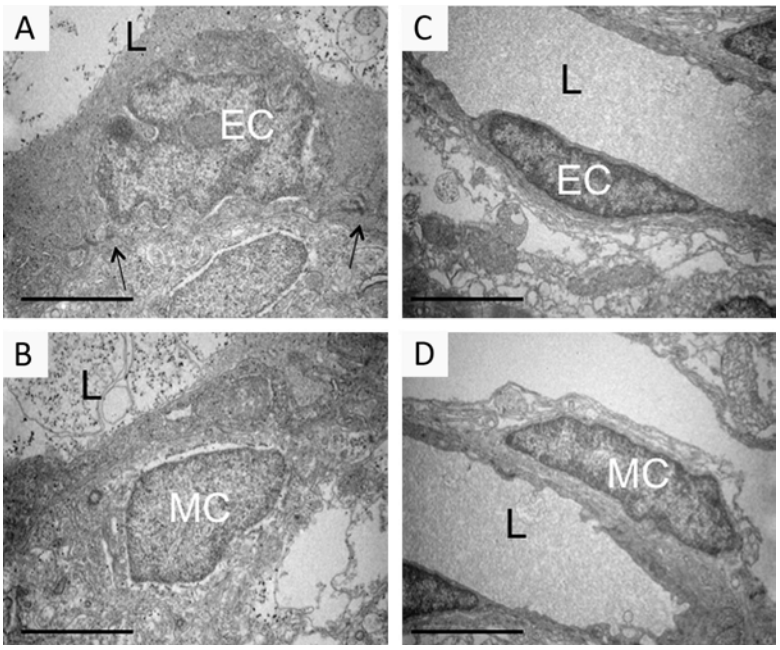


Figure 3: Vascular mural cells in zebrafish
Transmission electron micrograph of single mural cell (MC) surrounding endothelial vessels in 80 (A, B) and 120 (C, D) hpf larvae. Mural cells and endothelial cells (EC) exhibit different morphologies, and tight junctions are evident between ECs (black arrows). L, lumen. Scale bars are 5 µm. (modified from Santoro et al., 2009)

1.2.3 BLOOD FLOW WITHIN THE ORGANISM

Blood flow is generated through contraction of the heart. As arteries branch and lead to capillary beds in the tissues, the diameter of the vessels decreases. Because the number of vessels has increased, however, their combined cross-sectional area and circumference increase. As capillaries lead to veins and the veins coalesce into fewer and larger vessels, total cross-sectional area and circumference decrease. These changes have important functional consequences. A huge surface area is available in the capillary beds where exchange occurs. Also, because the total blood volume through the system at any give time is constant, the velocity of the flow changes greatly. It decreases as blood flows to the capillaries, and the

total cross sectional area increases. This results in more time for the exchange of oxygen and materials between blood and tissues. As blood is collected in the veins, flow rate increases again as the total cross-sectional area decreases. Blood pressure however, continues to decrease throughout the system due to friction (Satchell, 1991).

1.2.4 Additional roles for the vasculature

Apart from its crucial role in distributing oxygen, growth factors, and nutrients to developing organs and tissues, blood vessels have also been shown to play additional roles in processes such as neuronal migration and osteogenesis.

Vasophilic migration

An important characteristic of the development of the central nervous system (CNS) is the migration of neuronal precursors towards their sites of integration. Several migratory strategies can be employed for neuronal precursors to reach their target. The vast majority of neuronal precursors in the developing CNS migrate along radial glia processes (Saghatelyan, 2009). However, recent data suggest that neuronal precursors also use blood vessels for their navigation, at least in the adult mammalian brain (Bovetti et al., 2007, Snapyan et al., 2009). This is likely achieved through the secretion of migration promoting cues for neuroblast such as brain-derived neurothrophic factor (BDNF) by endothelial cells (Leventhal et al., 1999, Louissaint et al., 2002, Guo et al., 2008).

Blood vessels and osteogenesis

The vasculature is essential for embryonic skeletal development, bone growth, and remodelling. In addition to acting as a communicative network between bone and neighbouring tissues, the vasculature is essential in supplying the bone tissue with the necessary nutrients, growth factors, hormones, and osteoblast and osteoclast precursors as required (Kanczler and Oreffo, 2008, Saran et al., 2014). Moreover, vascularization is a prerequisite for osteogenesis to occur since the invasion of blood vessels promotes the chondroblast degradation of the cartilage core in long bones during endochondral bone formation (Gerber and Ferrara, 2000). Invasion of blood vessels is achieved through the expression of VEGF in osteoclasts, osteoblasts, and chondrocytes, which controls the timely invasion of endothelial cells and osteoclasts/chondroclasts into developing long bones during primary ossification (Cramer et al., 2004, Chim et al., 2013).

Angiogenesis has also been shown to be a key component in bone repair (Saran et al., 2014). Following a fracture, the resulting trauma, together with compromised blood supply, disruption of oxygen supply and acute necrosis of the surrounding tissue create a hypoxic environment. This hypoxic environment is an important regulator during bone repair as it regulates the expression of growth factors (e.g. HIF-1) in osteoblasts that in turn elicit the expression of osteogenic factors (e.g. BMP) by endothelial cells (Abidia, 2000, Malda et al., 2007). The ensuing inflammation and revascularization represents an essential phase during bone repair. Moreover, periendothelial cells (pericytes) have been shown to be capable of differentiating into osteoblasts, chondrocytes, adipocytes and fibroblasts (Diazflores et al., 1992, Doherty et al., 1998, Tintut et al., 2003).

1.3 THE NEUROVASCULAR LINK

Throughout the course of evolution, organisms have come to perform more and more complicated tasks requiring increased information processing by neurons and supply of nutrients by blood vessels. It is generally believed that the nervous system evolved prior to the development of the vascular system. First evidence of a nervous system under the form of diffuse nerve nets was detected in sea-dwelling organisms such as cnidarians. These organisms lack a vascular system since nutrients are readily available through diffusion (Miller, 2009). However, the increasing need for oxygen to fuel aerobic metabolism in multicellular organisms has driven the evolution of the vascular system (Fisher and Burggren, 2007). Despite the fact that both systems are functionally different, and that blood vessels arose later in evolution, they seem to share similar mechanisms and have co-opted similar molecular cascades that regulate their development. This cross-talk between vascular and neural networks is termed the neurovascular link, and can be inferred based on anatomical evidence and shared cellular, and molecular pathways (Carmeliet, 2003b, Carmeliet, 2003a, Carmeliet and Tessier-Lavigne, 2005, Zacchigna et al., 2008a, Segura et al., 2009).

1.3.1 ANATOMICAL EVIDENCE

At an anatomical level, the similar patterning between vessels and nerves can be readily observed, and could already be inferred from drawings made by Andreas Vesalius over five centuries ago (Figure 4). Recent studies in quail and mice have demonstrated the aligning of

blood vessels and nerves through wholemount histochemical staining, demonstrating the presence of large neurovascular bundles (Bates et al., 2002, Mukouyama et al., 2002, Larrivee et al., 2009). The ability of axons to guide blood vessels, and vice versa, is accomplished through the production of signal molecules (Carmeliet, 2003a, Carmeliet and Tessier-Lavigne, 2005). Such molecules, which affect both neural and vascular functions, have been recently termed angioneurins (Zacchigna et al., 2008b). For example, vascular smooth muscle expression of Artemin, part of the glial cell-derived neurotrofic factors (GDNF), attracts axons of sympathetic neurons (Honma *et al.*, 2002). Mice deficient in the production of Artemin exhibit aberrant neuronal patterning, demonstrating the capacity of vessels to guide developing nerves. Conversely, factors such as vascular endothelial growth factor (VEGF) are produced by neurons and Schwann cells to guide arteriogenesis (Mukouyama et al., 2002).

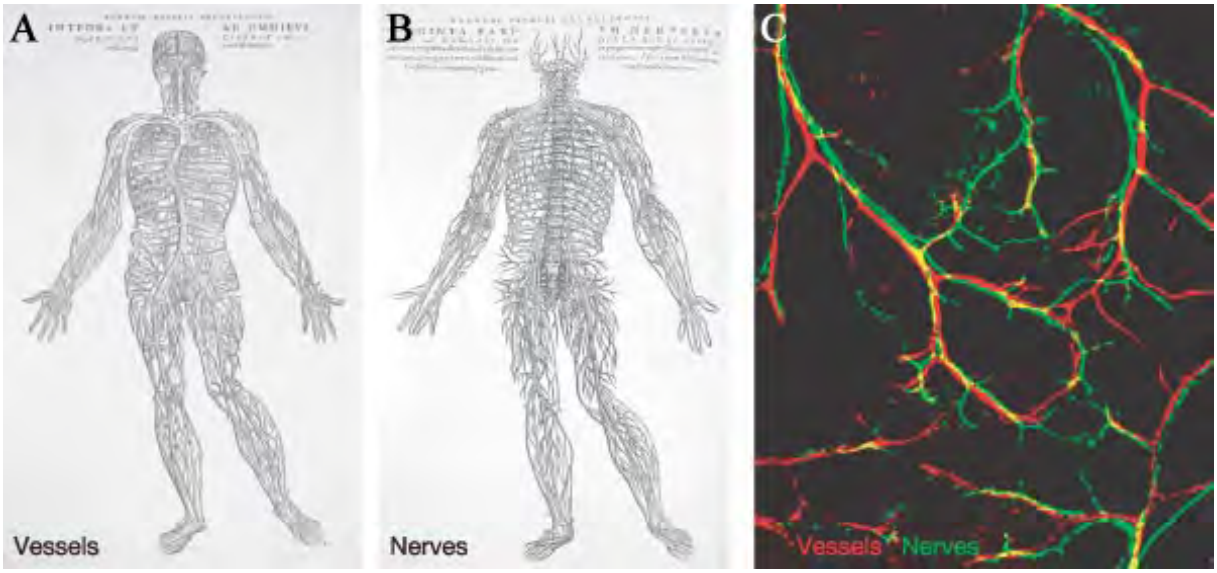


Figure 4: Parallels between vascular and neural networks
(A, B) Drawing of the Belgian anatomist Andreas Vesalius indicating the similarities in branching pattern between the vascular and the neural network in humans. (C) Vessels (red) and neurons (green) lining up together towards their targets in the skin of mice (reprinted from Carmeliet and Tessier-Lavigne, 2005).

1.3.2 SHARED CELLULAR MECHANISMS

Achieving the alignment of vessels and nerves requires the presence of sensory mechanisms that are able to detect the complex chemical cues present in the cellular environment. Superficially, both neural and vascular networks appear to develop differently. Neurons reach their target by sending out cable-like axons that migrate over considerable distances, whereas the development of vessels is mainly the result of local movements of endothelial cells (Carmeliet and Tessier-Lavigne, 2005). Nonetheless, both vessels and neurons possess similar specialized mechanisms to guide their development. In order to reach their target, axons are guided by a highly motile, sensory structure, called the growth cone (Figure 5A), which will continuously assess its spatial environment and select the proper route (Dickson, 2002). The growth cone is found at the end of an axon shaft and uses both actin-rich filopodia and lamellipodia, connected to the microtubule network, in order to advance towards its target (Yamada et al., 1970). In a similar manner, specialized cells appear to be present at the forefront of developing blood vessels, called endothelial tip cells (Figure 5B), which seem to share many similarities to axonal growth cones (Gerhardt et al., 2003). Hence, tip cells, similar to growth cones, are actin-rich structures that actively navigate through their environment, by use of both filopodia and lamellipodia, and guide the developing vessel towards its target.

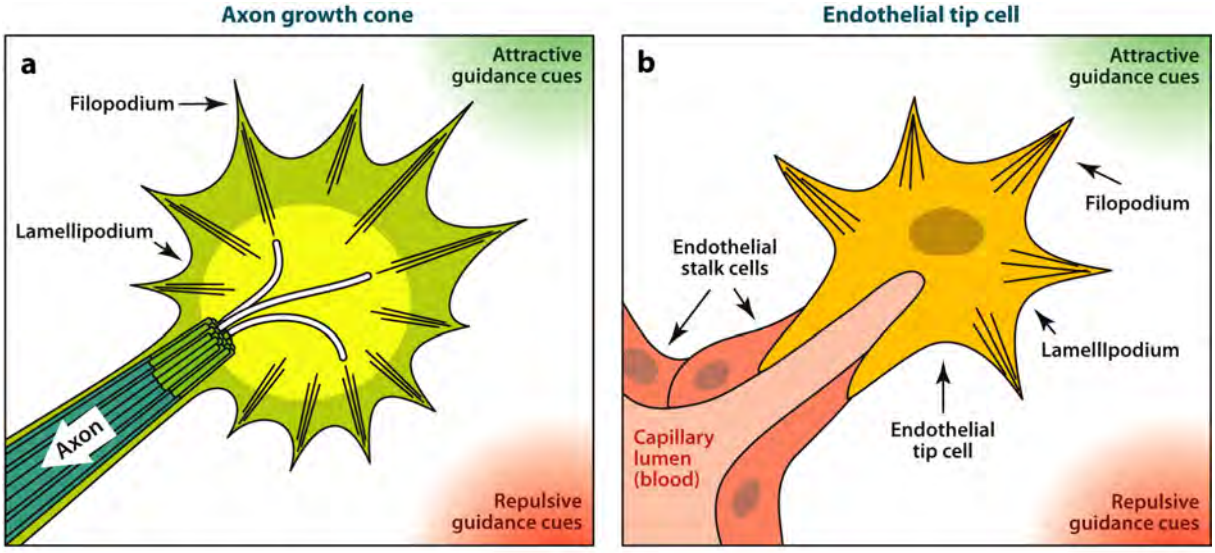


Figure 5: Axon growth cone versus endothelial tip cell
Schematic representation of an axon growth cone (a), and an endothelial tip cell (b). Both structures are functionally analogous and advance towards their targets through elaborate use of both filopodia and lamellipodia. Their development is guided through the presence of both attractive (green) and repulsive (red) cues in their cellular environment (reprinted from Tam and Watts, 2010).

1.3.3 SHARED MOLECULAR PATHWAYS

The similarities between axon growth cones and endothelial tip cells mentioned before suggest that they might respond in a comparable way to chemical cues present in their respective environments. Likewise, the stereotypical pattern of both the vascular and nervous system suggests the presence of common molecular cues to guide their development. In this regard, the classical axon guidance cues have been shown to be involved in the wiring of the vascular system. Axon guidance cues belong to four major families: (1) Slit/Robo, (2) semaphorin/plexin/neuropilin, (3) Netrin/Unc5/DCC, and (4) Ephrin/Eph (Figure 6). Their role during axon development has been extensively studied and is well established (Nieto, 1996, Tessier-Lavigne and Goodman, 1996, Dickson, 2002, O'Donnell et al., 2009). Their involvement in the wiring of the vascular system will be discussed below (reviewed in Tam and Watts, 2010).

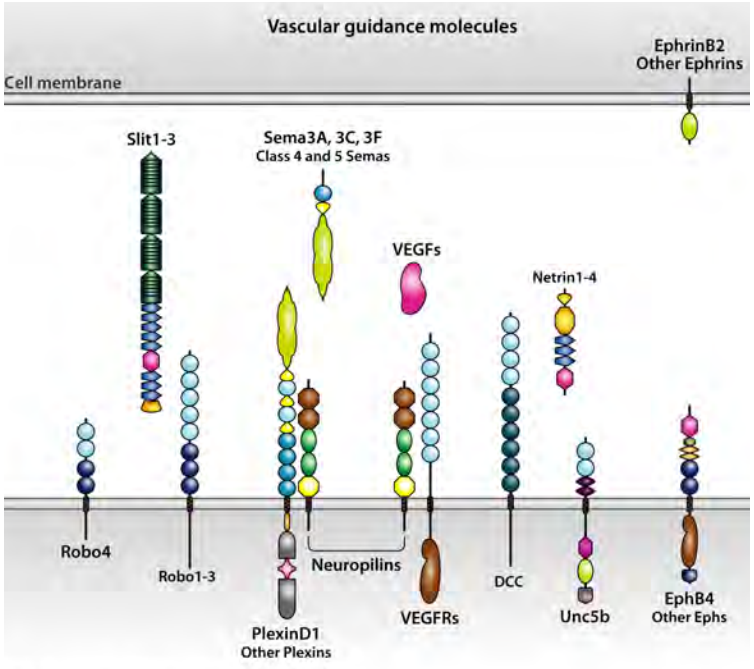


Figure 6: The major axon guidance cues with vascular effects
Schematic representation of the four major families of axon guidance ligand-receptor pairs:
- Slit/Robo
- semaphorin/plexin/neuropilin
- Netrin/Unc5/DCC
- Ephrin/Eph

These guidance cues have been recently shown to be involved in the guiding of endothelial tip cells (reprinted from Tam and Watts, 2010).

(1) Slit/Robo

Secreted Slit proteins signal through Roundabout (Robo) family receptors. In vertebrates, there are three members of the Slit ligand family (Slit1-3) and four members of the Robo receptor (Robo1-4) (Figure 6) (Larrivee et al., 2009). During neural development, interaction between ligand and receptor results in either attractive or repulsive responses to developing

neurons. Robo1–3 are mainly expressed in the nervous system, whereas Robo4 is structurally different and selectively expressed in endothelial cells (Larrivee et al., 2009). Robo4 is expressed in growing vessels during development and adulthood. In zebrafish, silencing of Robo4 had resulted in vessel sprouting defects (Kaur et al., 2008, Legg et al., 2008). However, other studies have shown that the binding of Slit2 to Robo4 inhibits VEGF induced endothelial cell migration (Jones et al., 2008). This would indicate that the pro- or anti-angiogenic properties of Robo4 are dependent on the nature of its ligand. In order to fully understand the role and molecular mechanisms of the Slit/Robo family members in angiogenesis, further research is required.

(2) semaphorin/plexin/neuropilin

Over 20 semaphorins, both secreted and membrane-bound, have been discovered. Semaphorins have generally been accepted as repellents in axon guidance and neuronal cell migration, although semaphorin 3A can also function as a chemoattractant (Carmeliet and Tessier-Lavigne, 2005). They contain a highly conserved extracellular domain (sema domain) that mediates binding to their neuropilin (NRP) or plexin receptors (Larrivee et al., 2009). NRPs form signalling complexes with plexins, but can also function as co-receptor for vascular endothelial growth factor receptors (VEGFRs) (Deutsch, 2004). Thus, In addition to being semaphorin receptors, NRPs bind multiple VEGF isoforms and mediate distinct VEGF functions (Soker et al., 2002, Pan et al., 2007). In heart cells for example, sema6D promotes VEGFR2 signalling through the activation of plexinA1, which forms a complex with VEGFR2 (Catalano et al., 2009). Moreover, binding of sema4D to its receptor plexinB1 has been shown to induce endothelial cell migration *in vitro* and angiogenesis *in vivo* (Zacchigna et al., 2008a). Hence, the Sema/Plexin/NRP family is an excellent example of a group of molecules with diverse functions in both neuronal and vascular development.

(3) Netrin/Unc5/DCC

Three members of the netrin family (netrin1, -3 and -4) have been identified in mammals. These ligands interact with two canonical receptor families: the deleted colorectal carcinoma receptor (DCC) subfamily (consisting of DCC and neogenin) and the uncoordinated (UNC) 5 subfamily (consisting of UNC5A–D) (Larrivee et al., 2009). Netrins act as attractive or repulsive cues for neurons depending on the receptors and intracellular levels of secondary messengers (Larrivee et al., 2009). In the vasculature, netrins and their receptors have been reported to exhibit pro- and anti-angiogenic activities. UNC5B expression is upregulated in

actively growing endothelial cells, but downregulated during quiescence (Lu et al., 2004, Wilson et al., 2006). In addition, binding of Netrin1 to UNC5B has been shown to induce retraction of tip cell filopodia and vessel regression (Lu et al., 2004). However, further studies are required to elucidate this apparent dual role of netrins during angiogenesis.

(4) Ephrin/Eph

Both Eph receptors and ephrin ligands are non-diffusible factors. The Eph receptor tyrosine kinases constitute a large family of transmembrane proteins with a single cytoplasmic kinase domain that becomes activated in response to binding to their ephrin ligands. Based on their sequence similarities and preferred binding ligands, receptors are subdivided into EphA (interacting with ephrinAs) and EphB (interacting with ephrinBs) (Egea and Klein, 2007, Pasquale, 2008). In the nervous system, Ephs and ephrins play key roles in the establishment of proper neuronal connections, including axon guidance and synapse formation. These guidance molecules are laid down in spatial gradients to shape precise trajectories for the axonal growth cone (Pasquale, 2008). Blood vessels, on the other hand, have also been found to express several Eph and ephrin family members. For instance, EphB4 and ephrinB2 are essential for arterial–venous specification and vascular remodelling, and are both expressed in venous and arterial endothelial cells respectively (Kuijper et al., 2007). In addition, EphrinB2 has also been demonstrated to play a role in the assembly of the blood vessel wall and in establishing proper mural-endothelial cell interactions (Foo et al., 2006).

1.4 THE NEUROVASCULAR LINK DURING TOOTH DEVELOPMENT

1.4.1 THE NECESSITY OF VASCULARIZATION AND INNERVATION OF TEETH

In order to obtain a fully functional organ, teeth must be able to grow in the appropriate environment and eventually receive the correct vascularization and innervation to maintain their function. Given that the maximum diffusion rate of oxygen and nutrients in tissues is 200-300 μm for air-breathing organisms (Muschler et al., 2004, Kannan et al., 2005) and even less for water-breathing organisms (Satchell, 1991), nutrient supply to the cells within the tooth would be insufficient without the formation of capillaries. Moreover, not only the limited diffusion rate of oxygen and nutrients determines the necessity for vascularization, but also the size of the tooth itself. In zebrafish for example, first-generation teeth are not

vascularized (Huysseune and Sire, 1998, Sire et al., 2002). This is probably related to the small size of the first teeth (approx. 50 µm tall, 12 µm across) compared to later generation teeth in adults (approx. 350 µm tall) (Wautier et al., 2001). In addition, comparing first-generation teeth of *Polypterus senegalus*, a basal actinopterygian, to zebrafish emphasizes the connection between tooth size and vascularization even further, since first-generation teeth in *P. senegalus* are generally larger (approx. 90 µm tall, 35 µm across) and are indeed vascularized (De Clercq et al., 2014).

The importance of blood vessels and nerves has also been acknowledged in the field of regenerative dentistry. Revascularization of the construct after implantation remains one of the key challenges in tissue engineering (Novosel et al., 2011), even more so since intrapulpal vasculature displays some unique features that cannot be easily mimicked, e.g. arteriovenous anastomoses (Takahashi et al., 1982).

Apart from blood vessels, it is also of paramount importance that implanted organs receive the correct innervation. It has already been shown that correct innervation of implanted tissues leads to increased and better growth of the tissue (Suuronen et al., 2004). Without the correct innervation of implanted teeth, pain sensation and vasomotoric control can never be achieved. Current studies are therefore focusing on restoring the vascular supply and innervation to engineered teeth (Nait Lechguer et al., 2008, Keller et al., 2012, Kokten et al., 2014) in addition to identifying growth factors that could aid in this process (Griffith and Naughton, 2002, Chen et al., 2010).

1.4.2 DURING MAMMALIAN TOOTH DEVELOPMENT

Mammalian tooth development is characterized by the coordinated development of an epithelial invagination (called enamel organ) and a mesenchymal (neural-crest) derived dental papilla, progressing through bud, cap and bell stage (Jernvall and Thesleff, 2000, Thesleff, 2003). The enamel organ differentiates into an outer and an inner dental epithelium, which surround the stellate reticulum and stratum intermedium. Epithelium-derived ameloblasts and mesenchyme-derived odontoblasts deposit enamel and dentine, respectively. The dental papilla eventually becomes the tooth pulp.

Most of the research in the past on vascular development during odontogenesis has been performed on molars and incisors of rats, teeth that are never replaced. These studies show the relatively late invasion of two independent vascular networks: one into the enamel organ and one into the dental papilla (Manzke et al., 2005). Vascularization of the enamel organ in rat

molars occurs when the tooth germ is fully developed, at the onset of dentinogenesis. Blood capillaries invade the stellate reticulum through the outer dental epithelium forming a complex, extensive network within the enamel organ. The outer enamel epithelium has been shown to exhibit gaps through which these capillaries can penetrate (Manzke et al., 2005). In a similar manner, a rich vasculature develops in the odontogenic zone of the dentine-pulp complex during primary dentinogenesis. In the mature tooth, the capillary network is limited to the subodontoblastic region (Yoshida and Ohshima, 1996). Apart from its role in (late) tooth development, angiogenesis is also essential for the successful repair and healing in the tooth. This process is possibly mediated by the release of growth factors, such as vascular endothelial growth factor (VEGF), contained within the dentine matrix (Roberts-Clark and Smith, 2000). Finally, vascularization is also essential for tooth eruption and replacement.

In order to perform their lifelong function, teeth also need to be richly innervated. The sensory input of an adult mammalian mandibular tooth originates from the trigeminal ganglion, more specifically from the inferior and superior alveolar nerve (Hildebrand et al., 1995, Fried et al., 2000, Magloire et al., 2010). Prospective dental nerves enter the jaw long before tooth formation, and a plexus of nerve branches is present in the mesenchyme beneath the thickened oral epithelium (Hildebrand et al., 1995). The pioneer dental axons reach the developing tooth germ at the bud stage, and subsequently, during epithelial folding morphogenesis, the nerve fibres innervate the dental follicle target field area. Surviving nerve fibres from the dental follicle penetrate the dental papilla during crown calcification (Pearson, 1977, Kollar and Lumsden, 1979, Luukko et al., 2005a, Luukko et al., 2008). In addition, the tooth pulp receives a sympathetic and, to some extent, a parasympathetic nerve supply (Haug and Heyeraas, 2006). The shift from the primary to the permanent dentition involves remodelling and growth of tooth nerves. Axons enter the developing permanent tooth pulps concomitantly with the degeneration of the primary pulpal axons (Hildebrand et al., 1995).

Evidence for a neurovascular link in mammalian tooth development has been suggested based on a close developmental association between blood vessels and nerve fibres (Zmijewska et al., 2003), on physiological effects (Byers et al., 2003), for example nerve fibres can influence the amount of blood which is supplied to the pulpal cavity (Hildebrand et al., 1995), and on shared molecular cues, e.g. axon pathfinding through Sema3A signalling (Luukko et al., 2005b).

1.4.3 DURING TELEOST TOOTH DEVELOPMENT

There is a severe lack of knowledge with regard to the vascular and neural supply of zebrafish teeth. In teleosts, the first to fourth pair of branchial (gill) arches are supplied by the aortic arches branching off from the ventral aorta (Isogai and Horiguchi, 1997). Blood vessels that supply the tooth-bearing area on the fifth branchial have not yet been identified. Innervation of this arch is probably provided by branches of the vagus nerve, as is the case for other teleosts (Jonz and Nurse, 2008). However, information regarding nerve patterning in this region is completely absent. In addition, given their location deep in the pharynx, at the boundary with the oesophagus, teeth possibly receive innervation from the enteric nervous system as well. Enteric neuron precursors arise from the vagal neural crest, migrate through the branchial arches, first into the anterior digestive system, and next populate the entire intestine (Olden et al., 2008).

In zebrafish, first-generation teeth are devoid of both vascular and neural elements, which is probably linked to the extreme small size of first-generation teeth compared to later generation teeth (Huysseune and Sire, 1998, Sire et al., 2002). Therefore, there are stronger indications for the presence of a neurovascular link in replacement teeth than for first-generation teeth. A role for nerve fibres in replacement tooth formation, on the other hand, may be inferred from an experiment conducted by Tuisku and Hildebrand (1994) who demonstrated the failure of tooth replacement in the lower jaw of the cichlid fish *Tilapia mariae* after transection of the trigeminal nerve. Moreover, similarities of teeth to other cycling organs that develop through epithelio-mesenchymal interactions, such as hairs (Pispa and Thesleff, 2003), further support the hypothesized role of neurovascular factors in tooth replacement. For example, nerve growth factor (NGF) and other neurotrophic factors show hair-cycle dependent changes (Zhou et al., 2006), and the vasculature surrounding the hair follicle undergoes hair-cycle dependent expansion and degeneration (Zhou et al., 2009).

1.4.4 A POSSIBLE ROLE FOR VEGF

VEGF is a secreted protein that acts via VEGF receptor signalling to regulate downstream targets. It has been shown to be a key regulator of angiogenesis in health and disease (Senger et al., 1983, Leung et al., 1989). Apart from its critical role in vascular development by stimulating division and migration of endothelial cells (Carmeliet et al., 1996b), it also appears to be involved in several neurobiological processes such as growth cone movement, neural survival, and maintenance of neural circuits (Zacchigna et al., 2008b). Thus, VEGF can

be rightfully called an angioneurin. Interestingly, in the light of a possible neurovascular influence on tooth development and replacement, it has already been detected in ameloblasts and stellate reticulum of human tooth germs as well as in odontoblasts (Mastrangelo et al., 2005, Miwa et al., 2010), suggesting that it might be involved in attracting blood vessels and nerves to the developing tooth.

VEGF ligands

There are several subtypes and isoforms of VEGF ligands and VEGF receptors. In humans, 5 VEGF family members - VEGFA, VEGFB, VEGFC, VEGFD and PlGF (Ferrara et al., 2003) have been identified, whereas in zebrafish only two VEGFA orthologues are known. The importance of VEGF family members in blood vessel formation is supported by evidence from VEGF gene knockouts in mice where both heterozygous (Carmeliet et al., 1996a, Ferrara et al., 1996) and homozygous (Karkkainen et al., 2004) mice are embryonic lethal. VEGFA has several human isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) (Houck et al., 1991, Ng et al., 2001, Robinson and Stringer, 2001). Zebrafish expresses VEGFA orthologues from two separate genetic loci that each express two separate isoforms. VegfAa (chromosome 16) give rise to $VegfAa_{121}$ and $VegfAa_{165}$ (Liang et al., 1998, Liang et al., 2001) isoforms, and the VegfAb locus (chromosome 4) produces the isoforms $VegfAb_{171}$ and $VegfAb_{210}$ (Bahary et al., 2007). The zebrafish VegfA orthologues (VegfAa and VegfAb) control sprouting angiogenesis and vascular integrity (Liang et al., 1998).

VEGF receptors

VEGF binds to type III tyrosine kinase receptors (VEGFR1-3). In mammals, VEGFA binds to VEGFR1 (Flt1) and VEGFR2 (Kdr/flk1), VEGFB binds to VEGFR1. Both VEGFC and D bind to VEGFR3 (Flt-4). Of the three receptors, VEGFR2 and VEGFR1 are considered to be the most important for blood vessel formation and VEGFR3 signalling is primarily responsible for lymphangiogesis. Zebrafish has one VEGFR1 (Flt1), two VEGFR2 (Kdra and Kdrb) and one VEGFR3 (Flt4) orthologue.

In the past, there has been some confusion on the classification of zebrafish VEGF receptor orthologues. Initially, only one VEGFR2 orthologue was found (Flk1/Kdra) on chromosome 4. It later became apparent that there were two VEGFR2 orthologues in zebrafish – kdra (Chr4) and kdrb (Chr 20). Covassin et al. (2006) argued that kdra is more closely related to VEGFR1 phylogenetically and may have evolved as a functional orthologue of mammalian VEGFR2. Others argue that kdra actually represents a fourth class (Vegfr4) of vertebrate

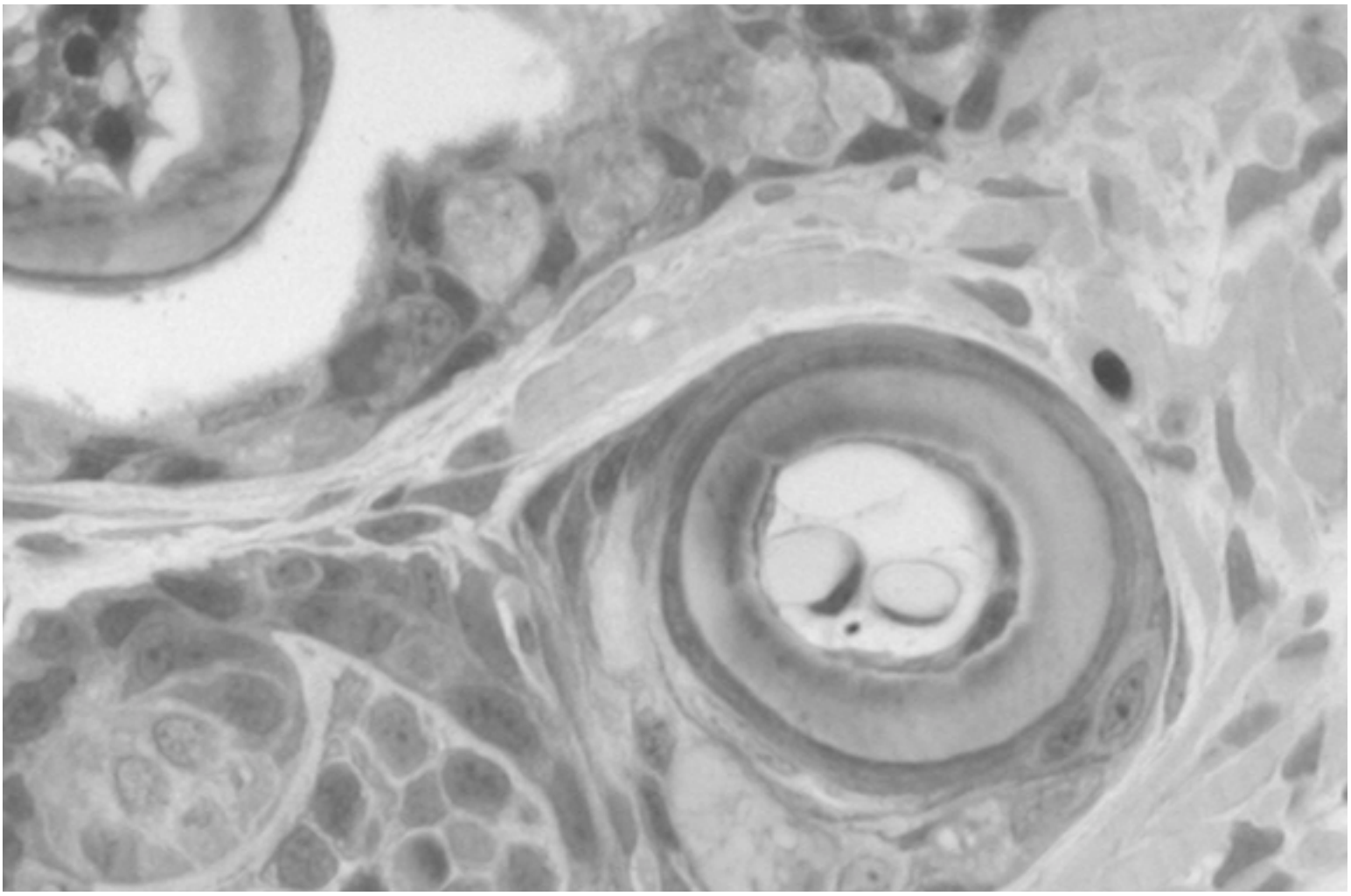
VEGF receptor and should therefore be annotated as vegfr4/kdr-like (kdrl) rather than flk1/kdra. According to the present nomenclature, there are four zebrafish VEGF receptors – Flt1/Vegfr1, Kdr/Vegfr2 (kdrb), Flt4/Vegfr3 and Kdrl/Vegfr4 (kdra) (Bussmann et al., 2008, Krueger et al., 2011, Wilkinson and van Eeden, 2014). Both Kdrl/Vegfr4 and Kdr/Vegfr2 are expressed in all endothelial cells and are activated downstream of VEGF (Bahary et al., 2007). During early vascular development in zebrafish – Kdr/Vegfr2, Flt4/Vegfr3 and Kdrl/Vegfr4 are coexpressed in most blood vessels. Kdrl/Vegfr4 is expressed from 12 hpf in the lateral mesoderm, is found in the forming dorsal aorta (DA) at 15- 29 hpf and is expressed in the axial vessels as well as inter-somitic sprouts by 24 hpf. Kdrl is required for sprouting angiogenesis during the formation of intersegmental vessels. Although Kdrl is expressed in the axial vessels, Kdrl knockdown affects angiogenesis - but not vasculogenesis or haematopoiesis (Habeck et al., 2002). Cytoplasmic or kinase domain mutations in Kdrl cause vascular development defects (Habeck et al., 2002). Kinase domain mutation (kinase dead mutant) causes partial dorsal sprout formation and abolishes dorsal longitudinal anastomotic vessel (DLAV) formation (Covassin et al., 2006).

Kdr/Vegfr2 has been shown to be expressed in dorsal sprouts, in the DA but not in the posterior cardinal vein (PCV). Although Kdr can partially rescue Kdrl/Vegfr4 loss of function, Kdr may not be required for vascular development (Covassin et al., 2006). Kdr knockdown leaves haematopoiesis unaffected. Combined knockdown of Kdr and Kdrl caused incomplete DA / caudal vein (CV) segregation, increases endothelial cell apoptosis and blocks formation of dorsal sprouts (Wiley et al., 2011).

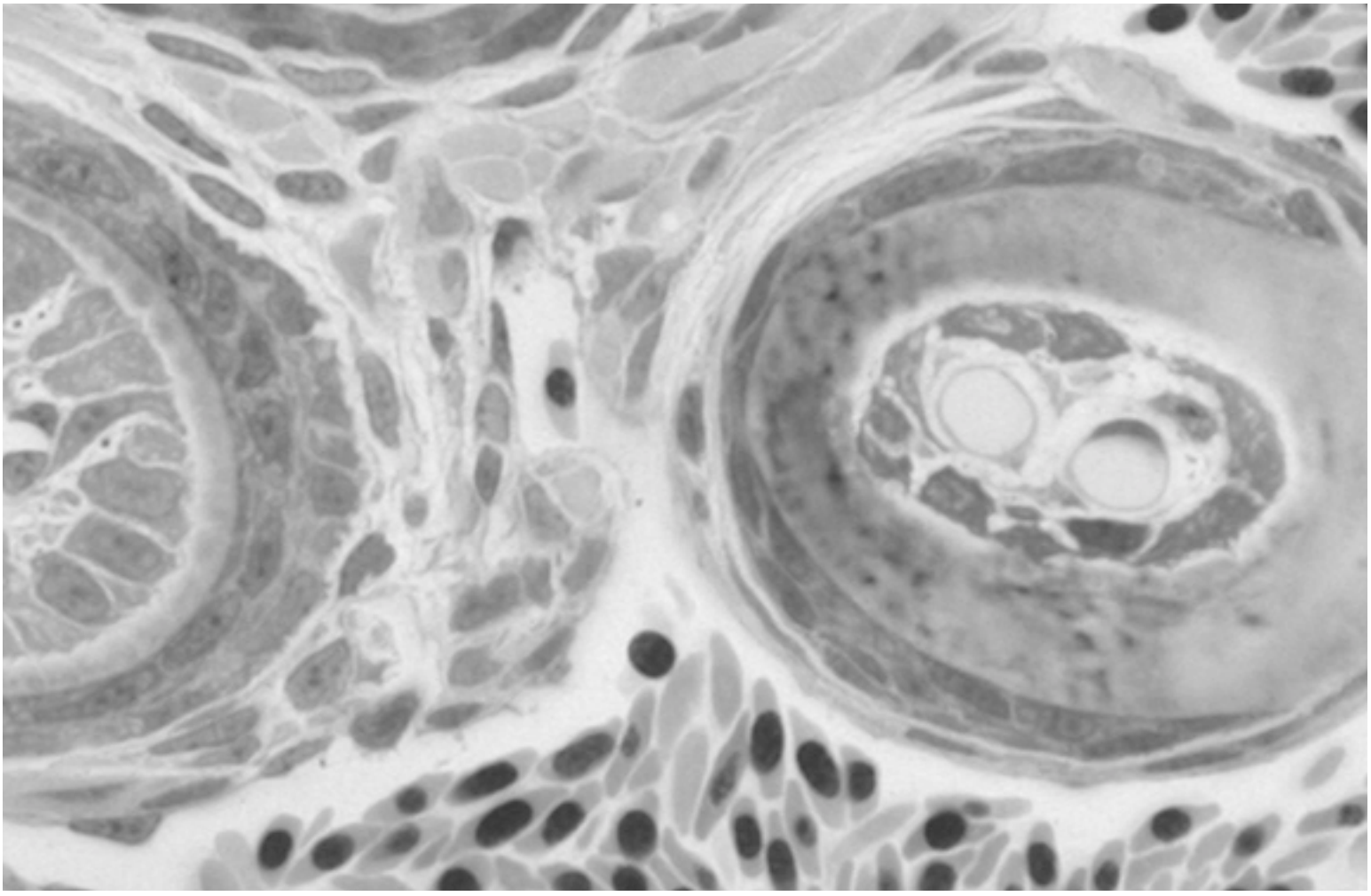
The zebrafish VEGFR3 orthologue Flt4 is located on chromosome 14 and produces one known transcript. At 24 hpf, the Flt4/Vegfr3 receptor is expressed in intersegmental sprouts and DA. DA expression subsequently becomes downregulated (Covassin et al., 2006, Hogan et al., 2009). Signalling through Flt4 does not seem to contribute to arterial sprout formation, but morpholino knockdown of Flt4 or Vegfc perturbs medial head vein formation (Covassin et al., 2006) which indicates that Vegfc - Flt4 signalling modulates venous rather than arterial angiogenesis. VEGFC knockdown in Kdrl/Vegfr4 mutants reduces intersegmental vessel expression of the arterial marker efnb2a, whereas loss of Flt4 in the same background does not affect efnb2a expression. Although VEGFC and VEGFR3 are thought to primarily control lymphangiogenesis rather than angio/vasculogenesis, VEGFC has also been shown to signal through VEGFR2 (Joukov et al., 1996) which could be explained by the fact that VEGFR1 can heterodimerize with VEGFR2 (Dixelius et al., 2003). Such pathway cross-talk has been demonstrated also in zebrafish (Covassin et al., 2006).

Chemical inhibition of VEGF signalling

Studying the effects of angiogenesis on the development of organs can be readily achieved by interfering with VEGFR downstream signalling, through application of pharmaceutical inhibitors. A wide array of compounds is available which have already been shown to block *de novo* development blood vessels. At present, pharmaceutical inhibition of angiogenesis is still an important component in the treatment of cancer (Carmeliet, 2005). Hence, attempts are still being made in identifying novel anti-angiogenic compounds (Senthilkumar et al., 2013, Lecht et al., 2015, Lin et al., 2015). A commonly used compound to block angiogenesis is called semaxanib (SU5416), a highly potent and selective inhibitor of the VEGFR2 by preventing VEGF-dependent autophosphorylation of the receptor. This compound is widely used as anti-angiogenic compound in both developmental and clinical research. In zebrafish, it is mainly used for testing its therapeutic properties in the treatment of cancer (Fong et al., 1999, Serbedzija et al., 1999, Mendel et al., 2000, Kuenen et al., 2002, Fiedler et al., 2003, Covassin et al., 2006). Besides the use of pharmaceutical compounds, a more recent approach in blocking angiogenesis goes beyond interference with VEGF signalling and focuses on suppressing the energy metabolism of the endothelial cells, thus preventing it from forming endothelial sprouts (Zecchin et al., 2014).



OBJECTIVES

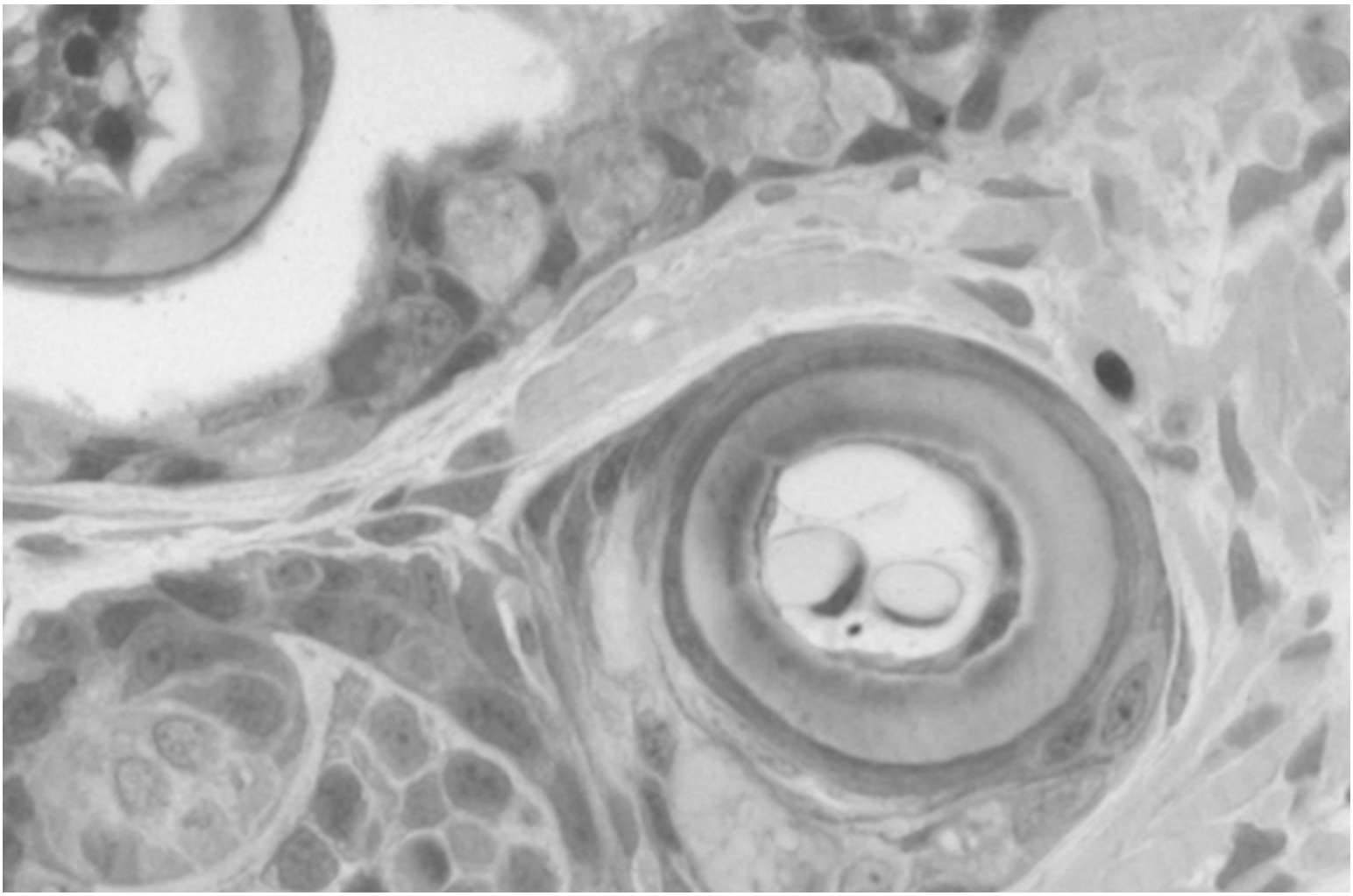


2. OBJECTIVES

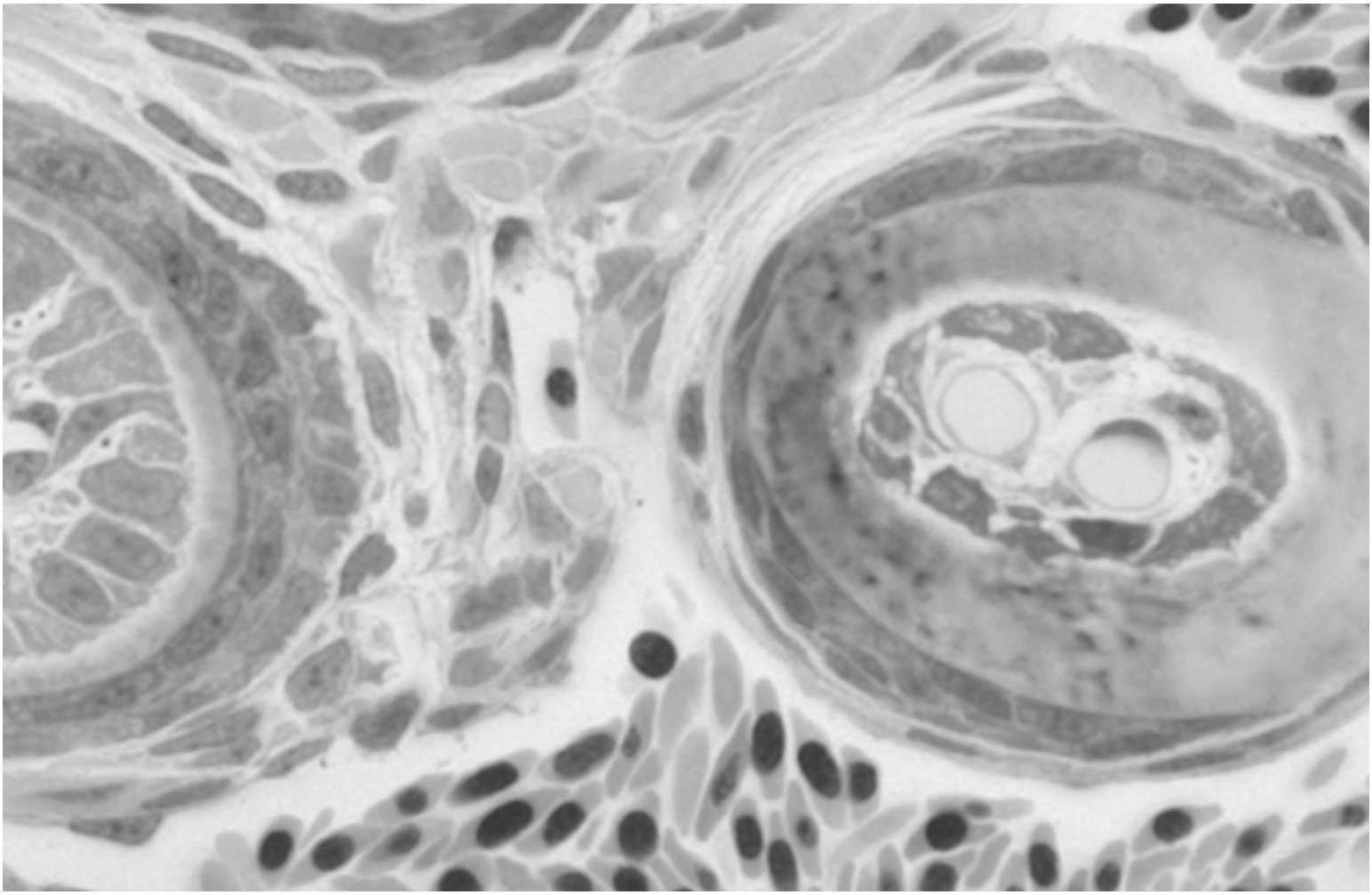
The role of angiogenesis and neurogenesis in tooth development and tooth replacement (and by extension in tooth engineering) has remained rather poorly studied. This is remarkable considering the vital role of both vascularization and innervation in promoting organ morphogenesis. Moreover, teeth are not isolated from their environment and require the correct innervation and vascularization in order to perform their lifelong function. Our aim is to test the hypothesis that tooth replacement depends on a properly functioning neurovascular link, using a model with natural *in situ* tooth replacement. To test this hypothesis a model system is used where (1) tooth formation (odontogenesis) is a repetitive event that occurs orchestrated in both time and space (Van der heyden and Huysseune, 2000), (2) tooth formation is sufficiently similar to odontogenesis in humans (Huysseune et al., 1998, Van der heyden et al., 2000), (3) genetic, molecular and developmental tools are available to study the respective roles of angiogenesis and neurogenesis in tooth formation. The zebrafish (*Danio rerio*) is a model that fulfils all of these requirements. In addition, the lack of both nerves and vessels in first-generation teeth of zebrafish (Sire et al., 2002), provides a nice negative control for treatments influencing either vascularization or innervation (e.g. to rule out possible toxic effects of pharmaceuticals).

(1) In the light of the current hypothesis we will use **a descriptive approach** in order to obtain anatomical evidence for a possible association between vascular and neural elements, on the one hand, and tooth formation on the other hand. We first examine the distribution of blood vessels and nerve fibres in the vicinity of developing primary (i.e., first-generation) teeth and replacement teeth.

(2) **A functional approach** will be used to identify angiogenic factors with a role in tooth development and replacement. A highly likely candidate is vascular endothelial growth factor (VEGF), an angiogenic factor that also appears to be involved in several neurobiological processes (therefore also termed an angioneurin). We examine the role of VEGF isoforms $VegfAa_{121}$ and $VegfAa_{165}$, during zebrafish tooth development by interfering with its signalling function.



RESULTS



3. RESULTS

OUTLINE

The following chapters comprise the results of this PhD thesis. Each chapter is formatted as a manuscript and deals with a certain aspect of the neurovascular link during tooth development and replacement in zebrafish.

The **first chapter** is a published manuscript in Journal of Anatomy entitled "Unravelling the blood supply to the zebrafish pharyngeal jaws and teeth." It concerns the description of a remarkable blood vessel pattern at the level of the teeth in zebrafish. In addition, we have elucidated the arterial blood supply to the fifth tooth bearing ceratobranchials. This research paper provides a firm morphological basis for future research focusing on the functional link between the vasculature and tooth development.

Chapter 2 entitled "The innervation of the zebrafish pharyngeal jaws and teeth." has been accepted for publication in Journal of Anatomy. The paper describes the way in which teeth are innervated in zebrafish. In addition, we have clearly demonstrated for the first time the presence of nerve fibres in the dental pulp of zebrafish.

The third and **final chapter** is a published manuscript in Journal of Dental Research entitled "Blocking VEGF signalling delays development of replacement teeth in zebrafish." Through application of semaxanib (SU5416), a vascular endothelial growth factor receptor inhibitor, we have studied the role of the blood vessels in the dentition of the zebrafish. We were able to show a delay in the developmental state of the replacer compared to what was expected based on the maturation state of the functional tooth. Hence, our results provide support for a nutritive, rather than an inductive, function of the vasculature in the process of tooth development and replacement.

Chapter 1

Unravelling the blood supply to the zebrafish pharyngeal jaws and teeth

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Key words: Zebrafish; blood supply; teeth; hypobranchial artery; vascularization

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1.1 ABSTRACT

We describe the vascular supply to the pharyngeal jaws and teeth in zebrafish, from larval stages to juveniles, using serial high quality semithin sections and 3D reconstructions. We have identified the arterial blood supply to the last pair of branchial arches, which carries the teeth, to issue from the hypobranchial artery. Surprisingly, the arteries supplying the pharyngeal jaws show an asymmetric branching pattern that is modified over ontogeny. Moreover, the blood vessel pattern that serves each jaw can best be described as a sinusoidal cavity encircling the bases of both the functional and replacement teeth. Capillaries branching from this sinusoidal cavity enter the pulp and constitute the intrinsic blood supply of the attached teeth. The role of these blood vessels during tooth development (whether instructive or nutritive) remains to be determined and requires further study. However, we have provided a firm morphological basis that will aid interpreting experiments that address this question.

1.2 INTRODUCTION

The zebrafish (*Danio rerio*) possesses numerous advantages (e.g. optical clarity of the embryo, ease of genetic manipulation, size, cost) that have rendered this small teleost an excellent model for the study of vertebrate development. Apart from its merits as a model organism for genetic, molecular, and developmental research (Lele and Krone, 1996, Roush, 1996), the zebrafish is also used as a model to study the processes of tooth development and continuous replacement (Huysseune et al., 1998, Van der heyden et al., 2001, Van der heyden et al., 2005, Verstraeten et al., 2010, Verstraeten et al., 2013).

Unlike most tooth-bearing vertebrates, the zebrafish has no oral teeth but has its teeth implanted on the ceratobranchials of the fifth branchial skeletal arch only (Huysseune and Sire, 1998, Wautier et al., 2001, Stock, 2007), also called pharyngeal jaws. Zebrafish possesses seven pharyngeal arches with arch three to seven corresponding to branchial arches one through five. Pharyngeal arches three through six (also termed branchial arches one through four) are gill-bearing, whereas pharyngeal arch seven/branchial arch five is not gill bearing but instead gives rise to the teeth.

The spatiotemporal pattern of tooth development and replacement in zebrafish has been well characterized. The zebrafish dentition consists of three tooth rows on each body side, which extend rostro-caudally: a ventral row (V) of five teeth (1V-5V), a mediodorsal row (MD) of four teeth (1MD-4MD), and a dorsal row (D) of two teeth (1D-2D) (Van der heyden and Huysseune, 2000). The first tooth bud already starts to develop at around 48 hours post-fertilization (hpf) at position 4V and is quickly followed by the development of the tooth germs at positions 3V and 5V. The teeth in the two rostral positions, 2V and 1V, develop at 12 and 16 dpf, respectively (Van der heyden and Huysseune, 2000). Replacement of first-generation teeth starts already at 80 hpf (position 4V) (Borday-Birraux et al., 2006). Contrary to first-generation teeth, replacement teeth do not arise directly from the pharyngeal epithelium, but rather from an epithelial invagination that develops from the crypt associated with the erupted predecessor tooth (Huysseune and Thesleff, 2004, Huysseune, 2006). This epithelial downgrowth is termed a successional lamina and is found in association with every functional tooth in the dentition. The distal end of this structure develops into a replacement tooth. The successional lamina itself is quickly taken up into the enamel organ of the developing tooth bud (Huysseune, 2006).

Blood vessels perfuse almost all tissues of the body and mediate critical exchange between tissues and blood. Moreover, endothelial cells have been shown to be involved in paracrine

signalling with surrounding organ cells (Cleaver and Melton, 2003). For example, in mice, endothelial cells have been shown to induce insulin expression in isolated endoderm, highlighting the importance of endothelial cells for pancreatic development (Lammert et al., 2001). In humans, perivascular cells have been identified as a source of mesenchymal stem cells (reviewed in Crisan et al., 2012). Moreover, in tissue engineering, revascularization is important to achieve proper development of the tissue (Jain et al., 2005, Nait Lechguer et al., 2008, Lovett et al., 2009). Hence, given the importance of the circulatory system in homeostasis, organ development, as well as in oxygen and nutrient supply, we can assume a role for blood vessels in tooth development and replacement. However, data regarding the vascular supply of teeth is scarce. Even in mammals, information is usually limited to the intrinsic blood supply of the tooth via the pulp cavity (Boyer and Neptune, 1962, Kindlova and Matena, 1962, Mattuella et al., 2007). Furthermore, studies that have focused on the role of blood vessels (whether instructive or permissive) in the process of naturally developing and replacing teeth are limited (Miller, 1957, Soderhol and Egelberg, 1973, Rygh et al., 1986). Recent studies have turned to studying the role of vascularization in engineered teeth (Nait Lechguer et al., 2008). The role of blood vessels in tooth development and replacement in the zebrafish has never been addressed. Not even the vascular supply to the tooth-bearing pharyngeal jaws has been identified. Hence, in order to study the function of blood vessels during tooth development we must first obtain a profound understanding of how blood is both supplied to, and drained from, the dentigerous area.

Two features, one biological and one technological, may greatly advance our understanding of the vascular supply of zebrafish teeth. First, teeth are implanted on the last pair of skeletal branchial arches, which represent serial homologues to the more anterior branchial arches. Second, the choice of the zebrafish offers great advantages over other model organisms for studies of vascular biology. Indeed, the introduction of the zebrafish, *Danio rerio*, as a model organism has opened new avenues in vascular biology research that have traditionally been much more difficult to study, for example vascular development *in vivo* (Zon, 1995, Isogai et al., 2001, Munoz-Chapuli, 2011). Numerous genetic and developmental studies have focused on the zebrafish circulatory network (Hu et al., 2000, Weinstein, 2002a, Kamei et al., 2004, Ellertsdottir et al., 2010, Ellett and Lieschke, 2010, Geudens and Gerhardt, 2011). As a result, a vascular atlas of the developing zebrafish embryo is now available. It demonstrates a strong similarity of the basic vascular pattern to that of other vertebrates, making the zebrafish an ideal model to study the genetic and molecular mechanisms regulating both angiogenesis and vasculogenesis (Isogai et al., 2001). In zebrafish, as in other teleosts, the cardiovascular

system is a simple loop consisting of the heart, arteries, and veins, with a gill capillary network intercalated to oxygenate the blood. The heart consists of four parts: sinus venosus, atrium, ventricle, and bulbus arteriosus. Deoxygenated blood is pumped via the sinus venosus into the atrium and subsequently into the ventricle, then passes through the bulbus arteriosus into the ventral aorta (Hu et al., 2000, Hu et al., 2001). Next, the blood enters the aortic arches (AAs). In larvae, blood flows posteriorly from the bulbus arteriosus into the ventral aorta, passing from the root of AA1 to AA6 (Isogai et al., 2001, Anderson et al., 2008). However, compared to larvae, in juveniles and adults the blood flows anteriorly from AA6 to AA3 (Hu et al., 2001).

Zebrafish possesses six pairs of AAs that connect the ventral aorta to the lateral dorsal aortae. AA1 develops at the onset of circulation and is a critical component of the initial circulation loop. In contrast, AA2 never fully develops and does not contribute to the final circulation. AA3 to 6 arise in branchial arch 1 through 4, respectively, and are called the branchial AAs since they will supply blood to the gills (Isogai et al., 2001). More specifically, AA3 and AA4 connect to the bilateral dorsal aortae, whereas AA5 and AA6 merge before connecting to the beginning of the single midline dorsal aorta (Olson, 2000a, Olson, 2000b, Isogai et al., 2001). In contrast, the fifth branchial arch does not bear gills in teleosts, and as a result is always neglected in descriptions of the vascular anatomy. Yet, in zebrafish, this is the tooth-bearing arch.

Here, we present a detailed morphological description of the spatial and temporal relationships between developing teeth, and their respective supplying arteries and draining veins in different life stages of the zebrafish. This description will serve as a reference for future research focussing on the functional link between the forming vasculature and the process of tooth replacement.

1.3 MATERIAL AND METHODS

Animal husbandry

Wild type zebrafish (*Danio rerio*) were bred and raised according to the methods described in Westerfield (1993). Briefly, fish were raised in a 14h/10h light/dark cycle at 28.5°C. Embryos were obtained via natural mating and raised in egg water. Embryos, larvae and juvenile fish were sacrificed according to the Belgian law on the protection of laboratory animals (KB d.d. 13 September 2004) by an overdose of MS222 (3-aminobenzoic acid ethyl ester). Embryos/larvae 2-6 days post-fertilization (dpf), and juvenile zebrafish with standard length 4.2, 4.6, 6.0, 6.1, 6.4, 7.3, 7.4, 8.0, 8.3, 9.5, 11 mm and 25 mm (ranging from 12 dpf to 75 dpf) were used. For early stages we use days post-fertilization since the fish were raised at standard temperature recommended for developmental studies (Westerfield, 1993). However, in older fish size variation becomes important and standard length gives a better indication of the developmental stage.

Tissue processing

Larvae and juvenile fish were processed according to Huysseune and Sire (1992). Briefly, they were fixed in a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde buffered with 0.2M cacodylate (pH 7.4) for 2 hours at room temperature. Juvenile zebrafish were decalcified by adding 0.1M EDTA to the fixative solution for one to several weeks at 4°C. The decalcifying solution was refreshed every two days. After fixation, animals were rinsed in 0.2M cacodylate buffer containing 10% sucrose, and postfixed for two hours at room temperature with 1% OsO_4 in 0.2M cacodylate buffer containing 8% sucrose. After rinsing in the same buffer, specimens were dehydrated through a graded series of ethanol and embedded in epon. Serial transverse and sagittal semithin (1 μm) sections were made using a Reichert-Jung ultra-cut ultramicrotome (Leica, Vienna, Austria). Sections were stained using toluidine blue, and mounted in DePeX (Gurr, BDH laboratory, UK). All sections were examined using a Zeiss Axio Imager Microscope and photographed using an Axiocam MRC videocamera. Reconstructions were made using the software program Amira® (v5.3.3.). Each reconstruction is based on a series of around 250 semithin sections in which the individual blood vessels were traced and labelled. Based on these labels the software program generates a 3D model. Finally, models were photographed from different perspectives using Rhinoceros® (v5.0).

Identification of blood vessels and nomenclature

To locate and identify blood vessels within the embryos and larvae we relied on the extensive work published by Isogai et al. (2001) giving a detailed description of the vascular anatomy of the developing zebrafish.

1.4 RESULTS

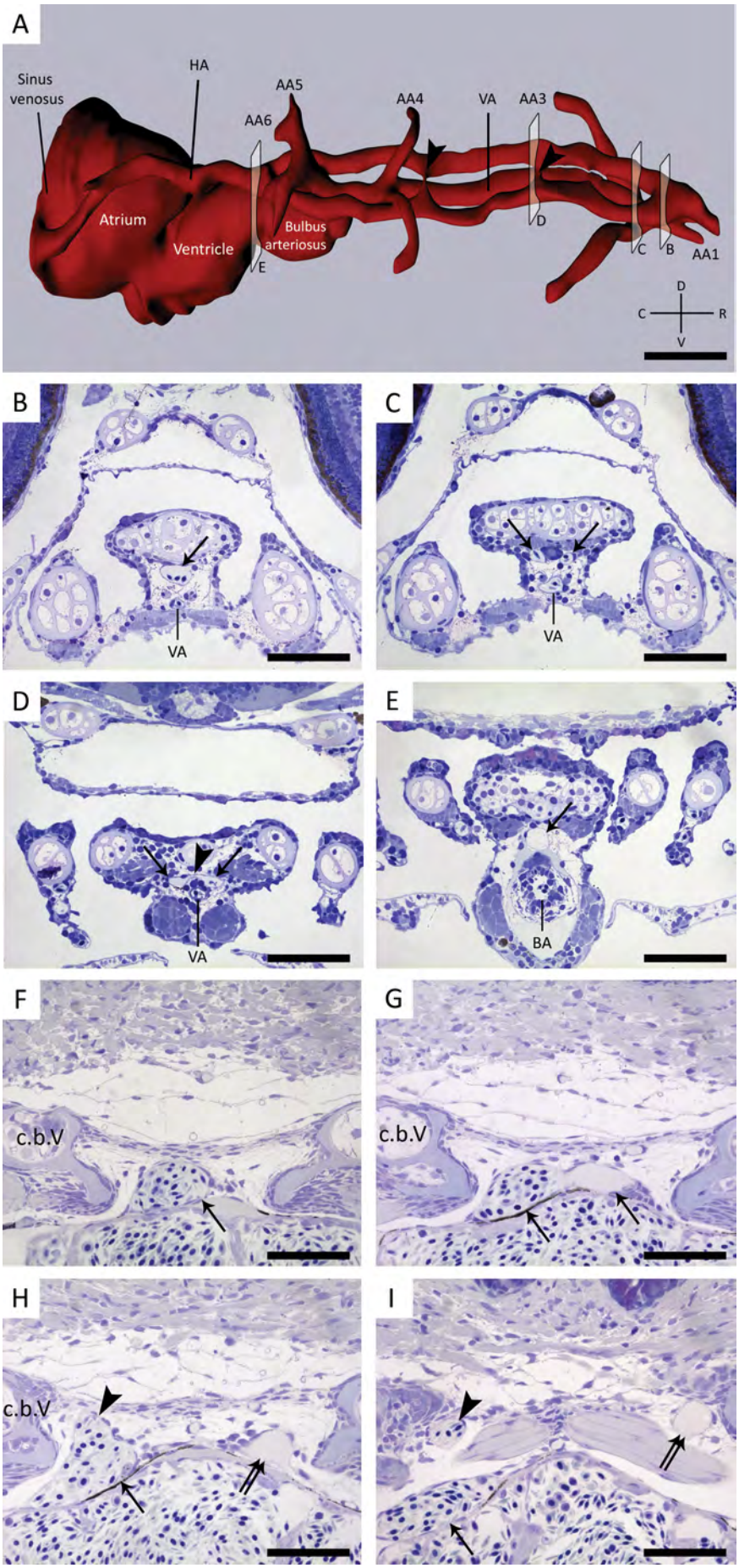
We will first focus on the arterial supply and venous drainage of the pharyngeal jaw region in zebrafish, and subsequently on the precise spatiotemporal relationship between blood vessels and the developing dentition.

Arterial blood supply to and venous drainage from the pharyngeal jaws

A 3D- reconstruction was made of the arteries supplying blood to the tooth-bearing region in a 6 dpf zebrafish, covering a distance of 220 μm along the anterior-posterior axis (Figure 7A). We identified the hypobranchial artery (HA) (Isogai et al., 2001), positioned just dorsal to the ventral aorta, as being responsible for securing blood to the teeth (Figure 7A). Starting from the cranialmost end of the ventral aorta, the HA bifurcates in two arteries (Figure 7B-C). Next, these paired vessels run posteriorly along either side of the ventral midline just dorsal to the ventral aorta (Figure 7A). At the level of the bulbus arteriosus, they merge again into a single artery (Figure 7E). Along their course, several connections exist between these paired arteries (Figure 7A, D). In a 26 dpf (SL = 6.1mm) fish we studied the HA in more detail at the level of the fifth ceratobranchials, where the most anteriorly positioned pharyngeal teeth are located. The HA is positioned just dorsal to the heart (Figure 7F). However, just caudal to the bulbus arteriosus, this vessel splits up in a smaller left and a larger right branch (Figure 7G).

Figure 7: Vascular supply to the zebrafish pharyngeal jaws

(A) 3D visualization of the vascular supply to the pharyngeal jaws in a 6dpf zebrafish showing the position of the hypobranchial artery (HA) with regard to the ventral aorta (VA) and the different aortic arches (AA1-AA6). (B-E) Transverse, toluidine blue stained sections of a wild type zebrafish (6 dpf) in the regions indicated in A. (B) Micrograph showing the position of the hypobranchial artery (arrow) dorsal to the ventral aorta (VA). (C, D) This artery bifurcates (arrows) and merges into a single unpaired median artery (arrow, E) at the level of the bulbus arteriosus (BA). Note the occasional connections (arrowheads in A, D) between the two vessels running along the ventral midline. (F-I) Transverse, toluidine blue stained sections of a wild type zebrafish (26 dpf, SL = 6.1 mm). (F) Micrograph illustrating the presence of the hypobranchial artery (arrow) in the vicinity of the tooth-bearing fifth ceratobranchials (c.b.V). (G) This artery splits in two branches (arrows) just caudal to the bulbus arteriosus. (H, I) Whereas the smaller branch on the left itself leads up to the teeth (double arrow), the larger branch on the right (arrow) gives rise to a dorsal offshoot (arrowhead), which serves the developing teeth of the right ceratobranchial. Further abbreviations: C = caudal, D = dorsal, R = rostral, V = ventral. Scale bars are 50 μm.


A
Sinus venosus
HA
AA5
AA6
AA4
VA
AA3
Atrium
Ventricle
Bulbus arteriosus
E
D
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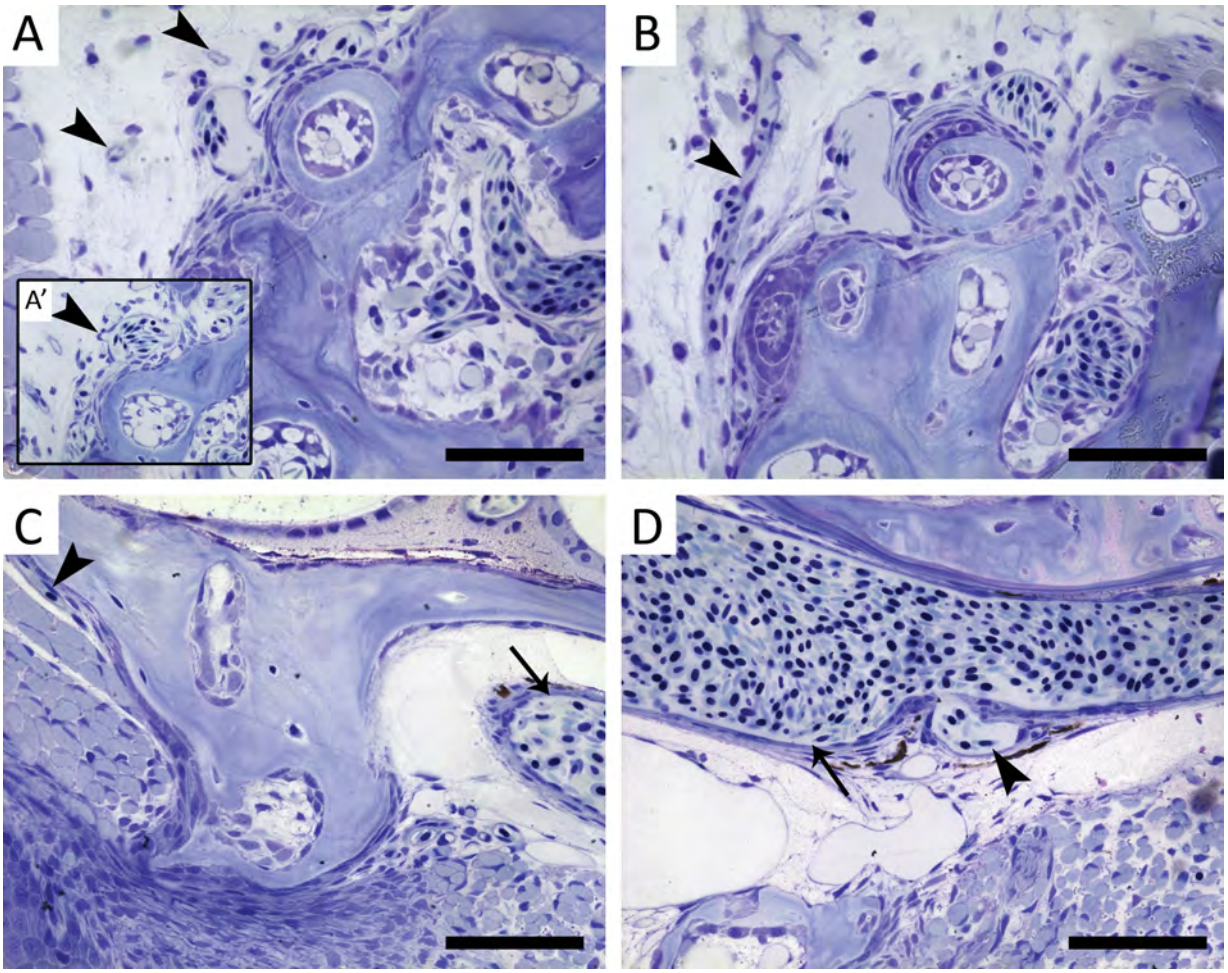
Detailed microscopical studies did not reveal any significant histological distinction between these two branches, such as differences in vessel wall architecture (data not shown). Immediately after this first split the larger branch on the right side gives rise to a dorsal offshoot (Figure 7H). Thus, whereas the smaller branch on the left body side is in itself responsible for the blood supply to the left fifth ceratobranchial, it is the dorsal offshoot of the larger right branch that reaches the teeth on the right side (Figure 7I). The main branch on the right side eventually connects to the heart through the sinus venosus (Figure 7A). Out of six individuals studied, with standard length 6.4, 7.3, 8.0, 9.5, 11 mm and one specimen 6 dpf, all of them (100%) displayed the same vascular pattern as described above.

Apart from identifying the supplying arteries, we also determined the way in which blood is drained from this region (Figure 8). The main branch on the right side itself connects to the sinus venosus and drains directly into the heart (Figure 8A). The blood that supplies the teeth is drained via small capillaries positioned on the lateral posterior side of the fifth ceratobranchial (Figure 8A). These capillaries branch off from the sinusoidal vessels (Figure 8A') surrounding the teeth (see below), and merge into a single vessel (Figure 8B). This vessel connects to the lateral dorsal aorta, just anterior to where the latter merges with its contralateral counterpart to form the single dorsal aorta that runs posteriorly along the body axis (Figure 8C-D). Hence, left and right pharyngeal jaw drain to bilateral vessels that each merge with their ipsilateral dorsal aorta, caudal to where AA5 and AA6 merge.

Figure 8: Drainage of blood from dentigerous region

Sagittal, toluidine blue stained sections of a wild type zebrafish (SL = 9.5 mm). (A) Small capillaries (arrowheads) can be seen at the lateral posterior side of the dentigerous area in close proximity to the sinusoidal cavity. (A') These capillaries have been observed to branch off from the blood vessels surrounding the teeth (arrowhead). (B) These smaller capillaries merge into a single larger vessel (arrowhead). (C) Next, this vessel (arrowhead) runs mediodorsally towards the position of the dorsal aorta (arrow). (D) Finally, it connects (arrowhead) to the lateral dorsal aorta (arrow). In all sections anterior is to the right. Scale bars are 100 µm.



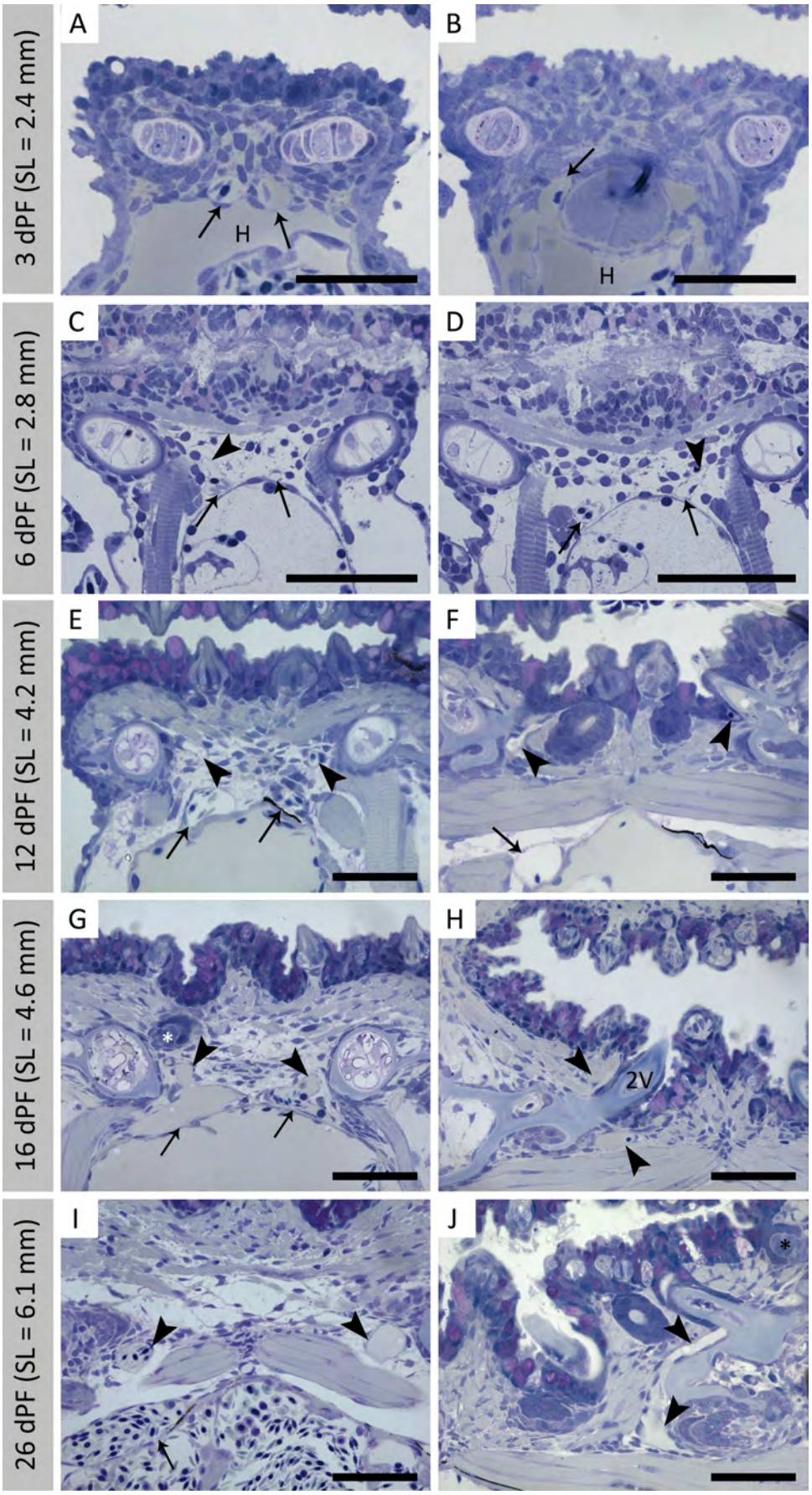
Spatiotemporal relationship between blood vessels and teeth

To identify the time at which blood vessels reach the dentigerous area during ontogeny, we studied wild type zebrafish of increasing age, two individuals each at 3 and 6 dpf (2.4 and 2.8 mm, resp.), and three individuals each at 4.2, 4.6 and 6.1 mm SL (Figure 9). Individuals of the same age displayed a similar vascular pattern. As stated before, blood is provided to the fifth tooth-bearing ceratobranchials via the HA. First evidence of this blood vessel can be observed at 3 dpf. At this stage, the bifurcation into the two branches serving each of the paired pharyngeal jaws is already present (Figure 9A). However, the dorsal offshoot on the right branch is still missing. The main branch itself on the right side already connects to the heart (Figure 9B). At 6 dpf, the dorsal offshoot on the right side of the body can be observed (Figure 9C). In addition, contrary to what we expect from the juvenile condition, we find the presence of a dorsal offshoot on the left side as well (Figure 9D). Nonetheless, the main branch on the left side is less pronounced than the one on the right and does not connect to the

sinus venosus. At this age three functional teeth ($3V^1$, $4V^1$, $5V^1$), and one replacement tooth ($4V^2$) are present. Blood vessels are completely lacking in the vicinity of these teeth. At 4.2 mm SL (12 dpf), the branches on both the left and right body side have dorsal offshoots that have reached the dentigerous area (Figure 9E). This is the anterior end of the sinusoidal cavity, as we will describe for the juvenile condition. Furthermore, at this stage, the bases of the functional teeth at position 3V, 4V and 5V have become encircled by blood vessels. Nonetheless, the dental pulp of first-generation teeth (i.e. teeth developing directly from the pharyngeal epithelium), for example the tooth at position 3V, does not appear to contain vascular elements. At 4.6 mm SL (16 dpf), each of the two branches of the HA still gives rise to a dorsal offshoot going towards the teeth (Figure 9G). Teeth that have become functional at this stage (e.g. at position 2V) are now encircled by blood vessels at their base (Figure 9H). Finally at 6.1 mm SL (26 dpf), the situation resembles what we observe in juveniles. The dorsal offshoot on the left side is all that remains; the main branch is no longer present (Figure 9I). Teeth that have become functional (e.g. at position 1V) are again encircled by blood vessels at their base (Figure. 9J).

Figure 9: Ontogeny of vasculature to teeth

Transverse, toluidine blue stained sections of wild type zebrafish 2.4 - 6.1 mm SL (3 - 26 dpf). (A, B) At 2.4 mm SL (3 dpf), both branches of the hypobranchial artery are present (arrows, A) while the right branch already connects to the heart (H) (arrow, B). (C, D) Next, at 2.8 mm SL (6 dpf), apart from the dorsal offshoot on the right branch (arrowhead, C), a dorsal offshoot can also be observed on the left side (arrowhead, D). Arrows indicate the two branches of the hypobranchial artery. (E-H) At 4.2 and 4.6 mm SL (12 and 16 dpf), blood is still supplied to the pharyngeal jaws via a dorsal offshoot of the branches on both sides of the body (arrowheads, E, G). Furthermore, starting from 4.2 mm SL onwards, blood vessels have reached the teeth and encircle the bases of the now functional, attached teeth (arrowheads, F, H). Both branches of the hypobranchial artery are still present (arrows, E, F, G). First-generation teeth, developing directly from the pharyngeal epithelium (asterisk, G), lack blood vessels in their immediate vicinity. (I, J) Finally at 6.1 mm SL (26 dpf), the dorsal offshoot is all that remains on the left side while the main branch is no longer present (right arrowhead, I). However, the main branch on the right side is still clearly visible (arrow, I). The sinusoidal cavity has become more pronounced encircling new teeth as they become functional (J, arrowheads). Scale bars are 50 µm.

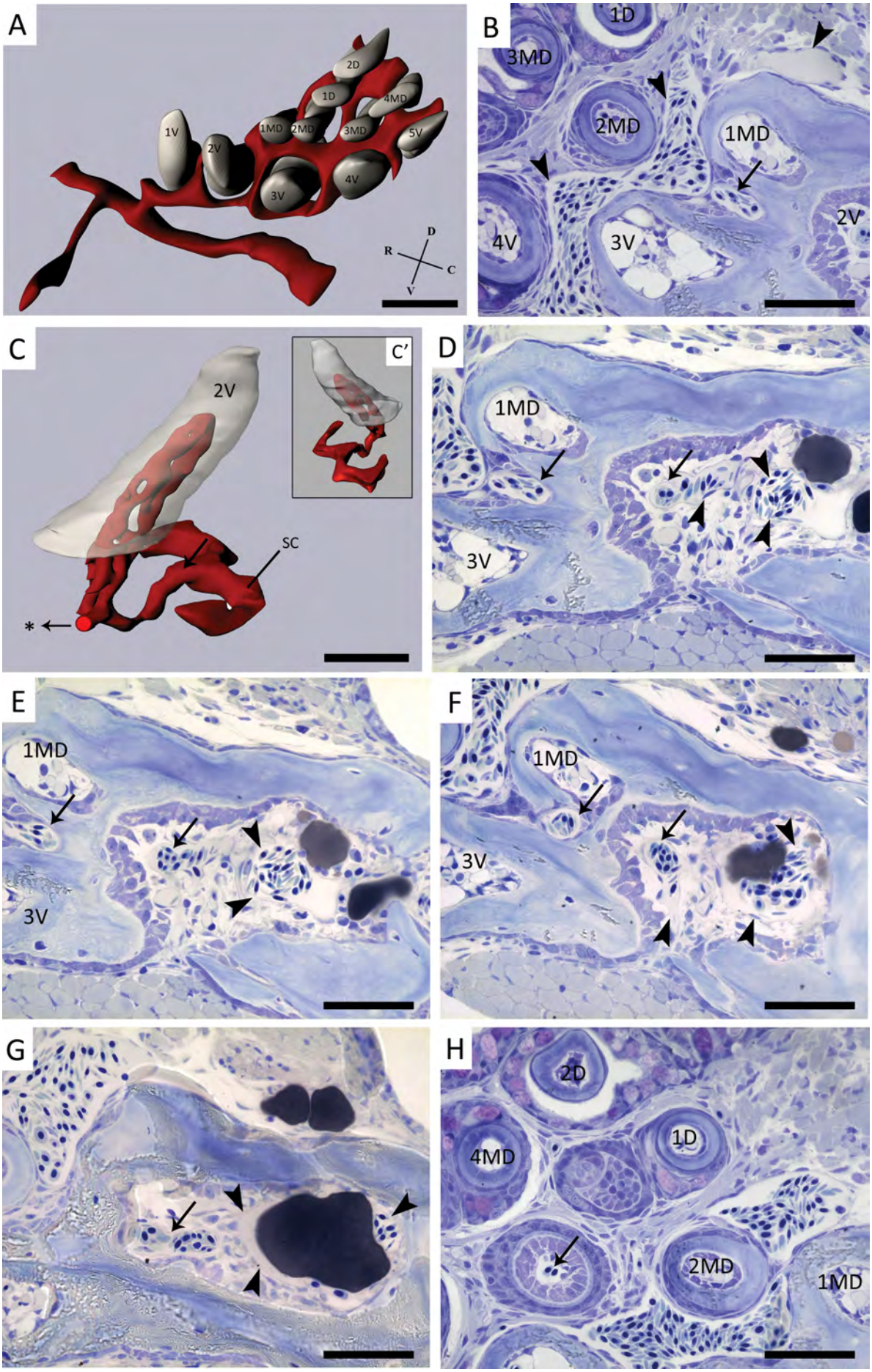

3 dPF (SL = 2.4 mm)
6 dPF (SL = 2.8 mm)
12 dPF (SL = 4.2 mm)
16 dPF (SL = 4.6 mm)
26 dPF (SL = 6.1 mm)
A
B
C
D
E
F
G
H
I
J
H
H
2V
*
*

In order to obtain a profound understanding of the spatiotemporal relationship between vascular elements and developing teeth, individual blood vessels were traced across serial semithin section of four different fish (SL = 6.4, 8.0, 11.0, and 25.0 mm). All specimens displayed the same pattern and a 3D reconstruction was made of the dentition and associated blood vessels of one of these fish (8.0 mm SL), covering a distance of 250 µm along the anterior-posterior axis. The resulting vascular pattern can be described as a sinusoidal cavity encircling the bases of both the functional and replacement teeth (Figure 10A).

The sinusoidal cavity on both sides of the body each connects to a branch of the hypobranchial artery, as described before. On each body side, a branch coming from this artery reaches the rostro-ventral side of the anteriormost tooth of the ventral row (1V), and is the beginning of a vessel, which bifurcates and anastomoses around the bases of the teeth, thus establishing a large, common sinusoidal cavity (Figure 10A). With the exception of tooth 3V, 2MD and 3MD, the teeth are never fully enclosed by this network of blood vessels.

Figure 10: Visualization of sinusoidal cavity and intrapulpal vessels

(A) 3D-reconstruction of a wild type zebrafish dentition (SL = 8.0 mm) illustrating the elaborate anastomosing network of vessels (red) around the bases of the developing teeth (grey). Note the organization of the teeth in a ventral (1V-5V), mediodorsal (1MD-4MD), and a dorsal (1D-2D) row. (B) Sagittal, toluidine blue stained section of a wild type zebrafish (SL = 9.5 mm) demonstrating the anastomosing network of blood vessels (arrowheads) surrounding the functional teeth (2V, 3V, 4V, 1MD, 2MD, 3MD, and 1D). Moreover, note the branch (arrow) issuing from the sinusoidal cavity, which is responsible for the intrinsic blood supply of the functional tooth at position 2V. (C) Detailed reconstruction of the tooth at position 2V showing the blood vessels (red) branching off from the sinusoidal cavity (SC) and entering the pulpal cavity. The arrow indicates the same branch as is highlighted in B. The exact course of the pulpal vessels, however, still remains unclear (*). (C') Different orientation of the reconstruction shown in C. (D-G) Sagittal, toluidine blue stained section of a wild type zebrafish (SL = 9.5 mm) illustrating the course of the branch issuing from the sinusoidal cavity (arrows, B-G)) prior to penetrating the pulp of the tooth at position 2V. Note the presence of several capillaries (arrowheads, D-G) merging with the branch from the sinusoidal cavity (arrows, B-G), preventing the complete identification of the course of the pulpal vessels. (H) Only replacement teeth in late cytodifferentiation stage appear to contain evidence of blood vessels penetrating the pulpal cavity (arrow). Black areas indicate the presence of lipids that are revealed through postfixation with OsO_4. Further abbreviations: C = caudal, D = dorsal, R = rostral, V = ventral. Scale bars are 50 µm.


A
1V
2V
3V
4V
5V
1D
2D
1MD
2MD
3MD
4MD
D
R
C
V
B
1D
3MD
2MD
1MD
2V
4V
3V
C
C'
2V
SC
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D
1MD
3V
E
1MD
3V
F
1MD
3V
G
H
2D
1D
4MD
2MD
1MD

Apart from the vascular elements surrounding the dentition, blood vessels can also be seen entering the functional teeth (i.e. teeth ankylosed to the ceratobranchial bone) at the tooth base. These blood vessels come from branches (Figure 10B) issuing from the sinusoidal cavity and are responsible for the intrinsic blood supply of the dental pulp. These branches are always issuing from the sinusoidal cavity at the postero-dorsal side of the tooth that they will penetrate. This was the case for all the teeth studied (n = 22) in a juvenile fish (SL = 9.5 mm). Reconstruction of vascular elements inside the pulp cavity of a tooth at position 2V visualizes the way in which these branches issuing from the sinusoidal cavity are responsible for the intrinsic blood supply of the teeth (Figure 10C). However, we could not fully track the course of the pulpal vessels due to the presence of several smaller capillaries merging with the branch issuing from the sinusoidal cavity at the base of the tooth, prior to penetrating the pulpal cavity (Figure 10D- G).

The dental organ does not appear to be vascularized. Indeed, of all teeth studied (n = 22), blood vessels were never observed in the dental organ.

Developing replacement teeth only appear to become vascularized during late cytodifferentiation/attachment stage (Figure 10H). Out of 22 teeth studied with their respective replacement teeth in different stages of development (i.e. initiation and morphogenesis, early and late cytodifferentiation, and attachment), only six appeared to contain blood vessels penetrating the pulp cavity (Figure 10H). All of these replacement teeth were already in late cytodifferentiation stage, close to attachment.

1.5 DISCUSSION

With this study we aimed at improving our understanding of the way in which teeth are vascularized in a small teleost, the zebrafish. On the one hand, we determined the arterial blood supply to the pharyngeal jaws, as well as their venous drainage. On the other hand, we elucidated the spatiotemporal relationship between the developing teeth and the surrounding blood vessels.

Arterial blood supply to the pharyngeal jaws

A median unpaired artery gives rise to branches that secure the blood supply to the pharyngeal teeth. Following the description of Isogai et al. 2001, together with our own microscopical observations, we have identified these branches as coming from the hypobranchial artery (HA) (Summarized in Figure 11). In short, a pair of HA sprout from the first AA, carrying oxygenated blood in a rostro-medial direction. Oxygenated blood reaches AA1 through the dorsal aorta from the third and fourth AA, which are the main AAs supplying the cranial circulation (Isogai et al., 2001). Next, the pair of HA merge again at the extreme rostro-ventral midline, continuing as a single vessel over a short distance in a caudal direction along the ventral midline. In front of the rostral end of the ventral aorta (VA), this vessel splits again into a pair of HA. The two vessels run posteriorly passing dorsal to AA1 and AA3, and ventral to AA4 through 6. In addition to what was already known about this vessel in zebrafish, we have established that prior to reaching the fifth ceratobranchials the pair of HA merges again into a single, median vessel. From then on, this vessel can be considered to be part of the coronary circulation, running over the surface of the heart providing it with oxygen, and finally connecting to the sinus venosus. This seems evident since, in mice, coronary vessels have been shown to arise from angiogenic sprouts of the sinus venosus (Red-Horse et al., 2010). In addition, previous studies have shown that the coronary circulation in zebrafish derives cranially from the hypobranchial artery (Hu et al., 2001). However, we have determined this vessel to give rise to branches supplying blood to the teeth, which has never before been observed.

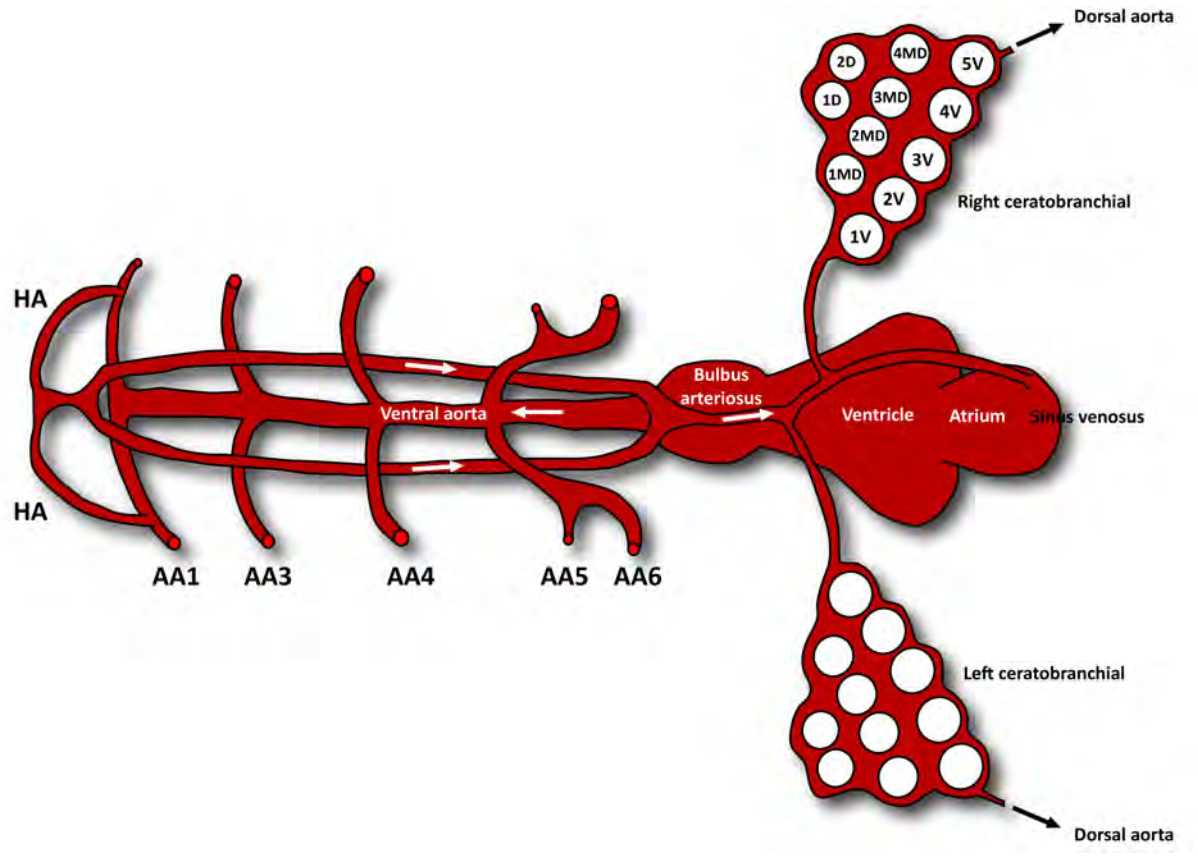

Figure 11: Schematic drawing of vascular supply to teeth
Schematic representation (dorsal view) of how blood is delivered through the hypobranchial artery (HA) to the tooth-bearing ceratobranchials in a juvenile zebrafish. AA1-AA6: aortic arches 1-6. Arrows indicate direction of blood flow. Note that this is only a schematic representation and does not fully correspond to what was observed since only teeth at position 3V, 2MD, and 3MD are fully enclosed by blood vessels.

There are some remarkable differences in the vascular anatomy in zebrafish compared to another teleost of which the vascular system has been well characterized, the rainbow trout (*Oncorhynchus mykiss*) (Isogai and Horiguchi, 1997) in particular with respect to the position of the hypobranchial artery. Whereas the HA in trout passes ventrally of the ventral aorta, the HA in zebrafish is found on its dorsal side. Furthermore, the HA in trout splits in two coronary arteries as it passes under the bulbus arteriosus (Olson, 2000a). In zebrafish too, the HA splits in two branches as it passes over the bulbus arteriosus. These two branches each give rise to dorsal offshoots running towards the teeth. However, at a size of 6.1 mm SL, the branch on the left is no longer present, leaving only the dorsal offshoot serving the teeth, and resulting in an asymmetric pattern of the arteries supplying the pharyngeal jaws. Thus, what appeared similar during development to the vascular plan in trout has become modified later in development, leaving only one coronary artery in zebrafish compared to two in trout. This

can be understood when comparing the physiology of both species. Compared to large, fast-swimming and predatory fish like trout, which usually possess extensive coronary vessels, the heart of smaller fish like zebrafish demands lower oxygen levels (Farrell et al., 2012).
Furthermore, we have also observed the presence of occasional connections between the two HA that pass just dorsal to the ventral aorta along the ventral midline (see Figure 7A). However, this was only observed in younger specimens (e.g. 6 dpf in Figure 7A), and never in juveniles. The vasculature in zebrafish is known to undergo extensive remodelling during development (Ellertsdottir et al., 2010, Herwig et al., 2011). Certain connections between blood vessels in zebrafish are known to become lost during development, while being replaced by new connections. This is similar to the formation of the intersegmental veins, where angiogenic sprouts coming from the posterior cardinal vein will connect to existing segmental arteries, transforming them in segmental veins (Isogai et al., 2003). The connections between the two HA can thus be seen as transient structures that will again disappear at a later developmental stage.

Venous drainage of the pharyngeal jaws

The coronary artery drains directly into the sinus venosus of the heart. The blood entering the sinusoidal cavity is collected through small capillaries that connect to the dorsal aorta just anterior to where the two lateral dorsal aortae merge into a single median vessel. This is analogous to the situation at the other branchial arches. Blood coming from the heart enters the gills through the afferent branchial arteries and connects to the dorsal aorta via the efferent branchial arteries (Olson, 2002, Skov and Bennett, 2005). However, different from what occurs at the other branchial (gill-bearing) arches, the fifth (tooth-bearing) arch delivers deoxygenated blood to the dorsal aorta. We can only speculate about the level of deoxygenation as the blood passes through the pharyngeal jaws. In rainbow trout, only 20 – 28% of the oxygen transported to the tissues of the avascular retina is consumed (Waser and Heisler, 2004). Likewise, we assume that the oxygen levels in the blood are only partially depleted after leaving the dentigerous area in the zebrafish. Hence, the remaining oxygen would become redistributed via the dorsal aorta to the other organs and tissues.

Spatiotemporal relationship between blood vessels and teeth

A 3D reconstruction has provided a clear visual representation of the spatial relationship between vascular elements and teeth. Capillaries branching from the sinusoidal cavity enter the pulp and constitute the intrinsic blood supply of the functional (i.e., attached and erupted)

teeth.

Comparatively little work has been conducted with respect to the vascular supply of teeth in common laboratory animals (rat, rabbit, hamster, opossum, monkey). Essentially, the blood supply of the teeth is considered to be coming from different sources, depending on the species (e.g. periosteal vessels, superior/inferior alveolar artery), and every tooth has its intrinsic supply which comprises a single or several smaller pulp vessels entering through the apical foramen (Perint, 1949, James, 1955, Cheng and Provenza, 1959, Adams, 1962b, Adams, 1962a, Boyer and Neptune, 1962). Similar to what we see in zebrafish, Cutright and Bhaskar (1969) have demonstrated the presence of a vascular plexus encircling the primitive tooth germs in the area of the dental sac in rhesus monkeys. Branches coming from this plexus enter the dental pulp and secure the intrinsic blood supply of the tooth. It is possible that in zebrafish, due to the arrangement of the teeth in three rows (rather than one as in rhesus monkeys), the vascular plexus of each tooth has anastomosed to generate one large sinusoidal cavity, giving off branches that will constitute the intrinsic blood supply of the teeth. However, we have yet to establish the exact course of the pulpal vessels.

Unlike in some other teleosts, we have not observed any evidence for capillaries penetrating the outer dental epithelium. In the Nile tilapia *(Oreochromis niloticus)*, an advanced teleost, many capillaries can be seen closely associated with the dental epithelium (Prostak et al., 1993, Sasagawa, 1997). These capillaries are most likely involved in active transport of inorganic ions (Garant, 1970) and fluoride (Suga et al., 1983) during formation, mineralization and maturation of enameloid. A possible reason for the distinctive distribution of blood vessels in the enamel organ in the two species could be the small tooth size and especially the small size of the enameloid cap in zebrafish compared to the Nile tilapia. Transport of ions through e.g. gap junctions (Huysseune and Sire, 1997) could be sufficient without involving the need for blood vessels.

The fact that the dentigerous area in zebrafish is so richly supplied with blood strongly suggests that the vascular system has an important role in the development and maintenance of the dentition. Possibly, the extensive vascularization may even be connected to the continuous replacement of the teeth. However, the sinusoidal cavity develops relatively late, and is not present when the first replacement tooth forms. In addition, blood vessels penetrating the pulp cavity have only been observed from second-generation teeth onwards, and only at the stage of cytodifferentiation, i.e. relatively late in tooth development. This suggests that the vasculature is likely not a trigger for tooth replacement as a process. Indeed, studies conducted in the past, albeit on mammals, have linked blood vessels to processes such

as tooth eruption and exfoliation, rather than tooth initiation (Hunter, 1778, Tomes, 1882, Massler and Schour, 1941, Miller, 1957, Kaku et al., 2001). Interestingly, a study conducted in mice and rabbits regarding the role of vascularization in tooth eruption and resorption, has demonstrated a connection between the number of osteoclasts and the degree in which the dental and surrounding tissues are vascularized (Miller, 1957). This supports a role for blood vessels in tooth resorption rather than tooth initiation. The larval teeth in zebrafish do not appear to contain pulpal vessels, confirming the observations already made by Sire et al. (2002) for zebrafish and other species, nor has the sinusoidal cavity developed yet. Still, these teeth are resorbed. This contradicts a role for blood vessels in tooth resorption. However, the observation that first-generation teeth persist and coexist with their successors for several generations (Van der heyden et al., 2000) might be linked to the lack of pulpal blood vessels in the larval teeth. The poor vascularization of first-generation teeth is probably connected to the small size of the dental pulp (approximately 2-3 cells wide), which is too small to accommodate vascular elements. Apart from a role in resorption, pulpal vessels can also serve a nutritive function. Later-generation teeth in zebrafish are larger in size compared to first-generation teeth thus increasing the need for nutrient and oxygen supply via the circulatory system. It is safe to assume that they require ingrowth of capillaries in order to grow beyond a certain size. To a certain extent, this dependency on blood vessels resembles the situation in growing tumours, which are not able to grow beyond a few millimetres without the ingrowth of capillaries from adjacent blood vessels (Folkman, 2001).

To determine the role of blood vessels in the process of tooth development and/or replacement, further research clearly needs to be done. For example, the study of vascular mutants or experiments involving the pharmaceutical blocking of vascular sprouts issuing from the sinusoidal cavity could help to elucidate the role of the vasculature in the dentition of zebrafish. Recent studies have shown the formation of supernumerary teeth in the zebrafish dentition, either when activating fgf signalling (Jackman et al., 2013) or by using retinoic acid (RA) treatments (Seritrakul et al., 2012). Exposure to exogenous RA has been shown to shift anteriorly the expression pattern of genes normally expressed in the tooth forming region (e.g. *pitx2* and *dlx2b*) causing a dramatic anterior expansion of the pharyngeal teeth (Seritrakul et al., 2012). These authors have linked this observation with a potential evolutionary role for RA in the dentition. In addition, the loss of fgf signalling in the oral epithelium has been hypothesized to be linked with cypriniform tooth loss (Stock et al., 2006). Both fgf and RA have already been shown to be involved in processes of blood vessel formation and are commonly targeted in cancer treatments to inhibit deregulated blood vessel formation (Cross

and Claesson-Welsh, 2001, Hoffmann et al., 2007, Kim et al., 2012). Thus, loss of teeth or presence of supernumerary teeth resulting from inhibition, respectively overactivation of these factors during tooth development, may well be mediated by the effect these factors have on the vasculature. Hence, it would be interesting to see whether these ectopic teeth are similarly vascularized (e.g. is there also a vascular plexus present?), and whether they can be replaced or not. However, lack of any published detailed histology of these supernumerary teeth prevents us to make any conclusion regarding their vascularization. Such studies may also shed light on whether vascularization plays a role in evolutionary changes in number, position or cycling of teeth. For example, Sutton and Graze (1985) have hypothesized that the hydrodynamic pressure caused by blood flow in the dental pulp and the tissues surrounding the tooth exerts a force towards the tooth and could cause it to move. Given the abundance of blood vessels in the dentigerous region as we have described here for zebrafish, hydrodynamic pressures as stated by the blood-vessel thrust theory (Sutton and Graze, 1985) could be involved in positioning of the teeth.

1.6 CONCLUDING REMARKS

We identified the blood supply to the pharyngeal jaws and revealed the presence of a remarkable vascular pattern serving the dentition in zebrafish. Having established this morphological baseline information, we can now engage in studies aiming to uncover the role of these blood vessels during tooth development and replacement. Based on the morphology, a role in initiation of replacement tooth formation seems unlikely, but this needs confirmation. The vasculature is probably more involved in tooth resorption and nutrition. However, further studies are required in order to reveal the role of the vasculature in a continuously replacing dentition, such as in zebrafish.

1.7 ACKNOWLEDGEMENTS

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Chapter 2

The innervation of the zebrafish pharyngeal jaws and teeth

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Key words: *Danio rerio*; vagal nerve; innervation; pharyngeal jaws; teeth; teleosts

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2.1 ABSTRACT

Zebrafish (*Danio rerio*) teeth are increasingly used as a model to study odontogenesis in non-mammalians. Using serial semithin section histology and immunohistochemistry, we have identified the nerves innervating the pharyngeal jaws and teeth. The last pair of branchial arches, which are non-gill bearing, but which carry the teeth, are innervated by an internal branch of a posttrematic ramus of the vagal nerve. Another, external, branch is probably responsible for the motor innervation of the branchiomeric musculature. Nerve fibres appear in the pulp cavity of the teeth only late during cytodifferentiation, and are therefore likely not involved in early steps of tooth formation. The precise role of the nervous system during continuous tooth replacement remains to be determined. Nonetheless, our study provides the necessary morphological background information to address this question.

2.2 INTRODUCTION

Over the last decades, extensive knowledge has been built up regarding the molecular mechanisms that govern tooth development (Jernvall and Thesleff, 2000, Thesleff, 2003, Jackman et al., 2004, Thesleff, 2006, Bei, 2009). However, teeth also need the appropriate environment providing the correct molecular and physiological cues to support development and growth. In particular, the presence of sensory and motor innervation is of vital importance for both the function and the protection of the tooth. Nonetheless, functional studies focussing on the role of nerves during tooth development and replacement are scarce (Lumsden and Buchanan, 1986, Harputluoglu, 1990, Tuisku and Hildebrand, 1994). This is remarkable given that the idea of a neuronal influence on the initiation of tooth development and replacement is not a new concept and has already been raised in the past (Pearson, 1977, Kollar and Lumsden, 1979, Lumsden, 1982, Chiego, 1995). This hypothesis is based on two observations. First, prospective dental nerves enter the mammalian jaw long before the development of teeth (Fried and Hildebrand, 1982). Second, early axons have a close spatial relationship to future sites of tooth development (Mohamed and Atkinson, 1983). Nevertheless, Lumsden and Buchanan (1986) have challenged this hypothesis through studies involving *in vitro* culture of mouse mandibular arch fragments, with or without trigeminal ganglion explants. Their results have led to the conclusion that tooth initiation does not involve a nervous component, at least during early steps of odontogenesis (Lumsden and Buchanan, 1986). However, a more recent study by Tuisku and Hildebrand (1994) in a polyphyodont teleost fish, *Tilapia mariae*, has demonstrated arrest of tooth replacement after denervation of the trigeminal nerve, hence providing evidence in favour of a neuronal influence on tooth development and replacement. In addition, a recent study in mice has also highlighted the possible importance of nerves during odontogenesis, by identifying peripheral nerve-associated glia as a possible source of multipotent mesenchymal stem cells that produce pulp cells and odontoblasts in the continuously growing incisor (Kaukua et al., 2014). These apparent conflicting results, and our wish to explore exactly how tooth replacement is initiated, have been the incentive for the current study. Using the zebrafish (*Danio rerio*), we have embarked on a study exploring the role of nerves during tooth development and replacement. However, prior to studying a functional relationship between nerves and developing teeth, we must first establish the morphological baseline information regarding the innervation of zebrafish teeth.

The zebrafish is a small teleost fish belonging to the cyprinids, and is widely used as a vertebrate model organism for genetic, molecular, and developmental research (Lele and Krone, 1996, Roush, 1996). Similar to most other tooth-bearing non-mammalian vertebrates, zebrafish replaces its teeth throughout life. The zebrafish has no oral dentition; its teeth are restricted to the pharyngeal region and are implanted on the fifth, i.e., last, branchial arch (Huysseune et al., 1998, Van der heyden et al., 2000, Stock, 2007). The complete pharyngeal dentition consists of three rows of teeth on each side: a ventral row (V) of five teeth (1V-5V), a mediodorsal row (MD) of four teeth (1MD-4MD), and a dorsal row (D) of two teeth (1D-2D) (Van der heyden and Huysseune, 2000). The first tooth bud starts to develop at around 48 hpf (hours post-fertilization) at position 4V (Van der heyden and Huysseune, 2000). The replacement of first-generation teeth starts already at 80 hpf (position 4V). First-generation teeth develop directly from the pharyngeal epithelium, whereas replacement teeth develop from an epithelial outgrowth at the base of the epithelial crypt surrounding the tip of the erupted functional tooth. This outgrowth is called the successional lamina (Huysseune, 2006).
The innervation of the teleost branchial arches has been described in several seminal papers and books (Goodrich, 1930, Grassé and Tetry, 1963, Nilsson, 1983, Beaumont and Cassier, 1994), albeit with inconsistent terminology and interpretation. In general, out of the 11 pairs of cranial nerves present, only number VII (facial), IX (glossopharyngeal), and X (vagus) are of major importance for branchial innervation, and are therefore called the branchial nerves (Nilsson, 1983, Nilsson, 1984, Sundin and Nilsson, 2002). These three nerves enter the branchial (or gill) arches dorsally and form large nerve trunks (Jonz and Nurse, 2008). The facial nerve (N.VII) projects efferent (motor) fibres to the most anterior pharyngeal arch, and receives afferent (sensory) fibres from the taste buds in the mouth region, gills, and in some species from large areas of the body surface (Laurent and Dunel, 1966, Ezeasor, 1982, Morita and Finger, 1985). The glossopharyngeal nerve (N.IX) receives sensory information from the pseudobranch and contains both sensory and motor fibres innervating the first gill arch (third pharyngeal arch). The vagal nerve (N.X) innervates the other gill arches with afferent and efferent fibres (Nilsson, 1984). Upon entering the branchial region, these branchial nerves subdivide into a pretrematic and posttrematic ramus that enclose the gill slits, rostrally and caudally respectively (Nilsson, 1983, Nilsson, 1984, Sundin and Nilsson, 2002). The pretrematic ramus carries sensory information, whereas the posttrematic nerve branch contains both sensory and motor fibres (Nilsson, 1984).
Since branchial arches one to four bear the gills, considerable information is available on their innervation (Goodrich, 1930, Grassé and Tetry, 1963, Nilsson, 1983, Nilsson, 1984, Dunelerb

et al., 1993, Beaumont and Cassier, 1994, Chandrasekhar et al., 1997, Sundin and Nilsson, 2002, Jonz and Zaccone, 2009, Young et al., 2011). Data regarding the innervation of the fifth arch is lacking, yet this is the arch that carries the pharyngeal teeth in zebrafish and in many other teleosts (Huysseune and Sire, 1997, Huysseune et al., 1998, Sire et al., 2002). Thus, there is also a complete lack of knowledge regarding the nervous supply of the teeth in zebrafish. In order to study the function of nerves during tooth development and replacement, we must first identify the nerves responsible for innervating the fifth ceratobranchials, establish whether or not the teeth themselves become innervated, and determine the precise timing of these events. We hypothesize that branches of the vagal nerve innervate the pharyngeal jaws and teeth, however, this needs to be confirmed.

2.3. MATERIAL AND METHODS

Animal husbandry

Wild type zebrafish (*Danio rerio*) were bred and raised according to the methods described in Westerfield (1993). Briefly, fish were raised in a 14h/10h light/dark cycle at 28.5°C. Embryos were obtained via natural mating and raised in egg water. Embryos, larvae, juvenile and adult fish were sacrificed according to the Belgian law on the protection of laboratory animals (KB d.d. 13 September 2004) by an overdose of MS222 (3-aminobenzoic acid ethyl ester). Juvenile zebrafish with standard length 6.0, 7.3, 8.0, 8.3, 9.5 mm and 11.0 mm, and five adult fish were used. All juvenile fish were used for light microscopical analysis, whereas the five adult fish were processed for immunohistochemistry.

Histology

Fish were fixed and embedded in epon following Huysseune & Sire (1992). Briefly, they were fixed in a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde buffered with 0.2M cacodylate (pH 7.4) for 2 hours at room temperature. Juvenile zebrafish were decalcified by adding 0.1M EDTA to the fixative solution for one to several weeks at 4°C. The decalcifying solution was refreshed every two days. After fixation, animals were rinsed in 0.2M cacodylate buffer containing 10% sucrose, and postfixed for two hours at room temperature with 1% OsO_4 in 0.2M cacodylate buffer containing 8% sucrose. After rinsing in the same buffer, specimens were dehydrated through a graded series of ethanol and embedded in epon. Serial semithin (1 μm) sections were made using a Reichert-Jung Ultra-cut ultramicrotome (Leica, Vienna, Austria). These sections were stained with toluidine blue, and mounted in DePeX (Gurr, BDH laboratory, UK). All sections were examined using a Zeiss Axio Imager Microscope and photographed using an Axiocam MRC video camera. Reconstructions were made using the software program Amira® (v5.3.3.). Finally, 3D models were photographed from different perspectives using Rhinoceros® (v5.0).

Tissue processing for immunohistochemistry

Adult fish were sacrificed using a lethal dose of 0.5% MS222 and the heads were fixed in 4% PFA for 48 hr. The heads were decalcified in Morse's solution (Morse, 1945) for 2-4 weeks and rinsed for 12 hr in running tap water. Subsequently, they were dehydrated in an ascending series of ethanol (30%, 50%, 70% ethanol, 12 hr each), and three times 2hr in 96% ethanol. Next, the heads were immersed in three baths of Ultraclear (J.T. Baker, Deventer, The

Netherlands) for 12 hr and subsequently in three paraffin baths for 8 hr at 60–70°C. Heads were embedded in peel-away moulds (Ted Pella, Inc., Redding (CA), USA). Transverse sections (5µm) were made on a MicromHM360 microtome (Prosan, Merelbeke, Belgium).

Immunohistochemistry and visualization

The slides with paraffin sections were placed on a hot plate (70°C) for 5 min, cooled down for 5 min, and heated again for 5 min. The paraffin was removed in two Ultraclear baths for 15 and 10 min, respectively, and the sections rehydrated in a descending ethanol series. The slides were next rinsed in 1xPBS/1% DMSO three times for 5 min. Blocking in 3% BSA/1% milk/1xPBS/1% DMSO for 2 hr at room temperature reduced the background signal. After blocking, the sections were demarcated with a hydrophobic pen (Dakopen, Heverlee, Belgium) to limit the amount of antibody needed. Per group of serial sections, one section was marked out and was incubated with pure block- solution to serve as a negative control. The monoclonal anti-acetylated alpha tubulin antibody produced in mouse (T5168, Sigma, Diegem, Belgium) was dissolved in block-solution at a 1/300 concentration. The antibody-covered slides were placed overnight in a wet chamber at 4°C. The slides were next rinsed in 1xPBS/1% DMSO three times for 5 min. The polyclonal anti-mouse antibody produced in goat (IgGDyLight 594- ab96881, Abcam, Cambridge, UK) was dissolved in blocking solution at a 1/300 concentration and was placed on the tissue for 2 hr at room temperature, sheltered from light. The tissue was rinsed with 1xPBS/1% DMSO and DAPI (1 mL/mL distilled water) was added for 5 min. Finally, the slides were rinsed with 1xPBS/ 1% DMSO three times for 5 min, and mounted with Vectashield (Vector Laboratories, Inc., Peterborough, UK). Immunofluorescence was visualized using a NIKON eclipse TE2000-S confocal laser-scanning microscope (Nikon, Melville (NY), USA).

2.4 RESULTS

The innervation pattern of the pharyngeal jaws and teeth was studied using both semithin sections and immunohistological staining of nerve fibres.

Vagal sensory ganglion and its branches

Study of toluidine blue stained sections of 8.3 mm SL zebrafish revealed the presence of a large ganglion on each body side. Based on earlier publications of the zebrafish nervous system (Higashijima et al., 2000, Holzschuh et al., 2005), and the position of the ganglion on the postero-dorsal side of the dentition, just lateral of the inner ear, and dorsal of the posterior cardinal vein (Figure 12A, B), we could identify this ganglion as the large sensory ganglion of the vagal nerve. For complete visualization of the ganglion and its different branches, a 3D reconstruction of 111 consecutive semithin (1 µm) sections was made. A total of six different branches could be identified (Figure 12C, D). Two branches emerge from a common root and course ventrally towards the dentigerous region. We termed the more anterior of these two branches internal (branch 2 on Figure 12C,D), and the more posterior one external (branch 1 on Figure 12C,D). Three separate branches run medially towards the pharyngeal epithelium, and a final, sixth branch extends caudally. The ganglion connects to the rhombencephalon more antero-dorsally (data not shown). These findings were confirmed in three different specimens.

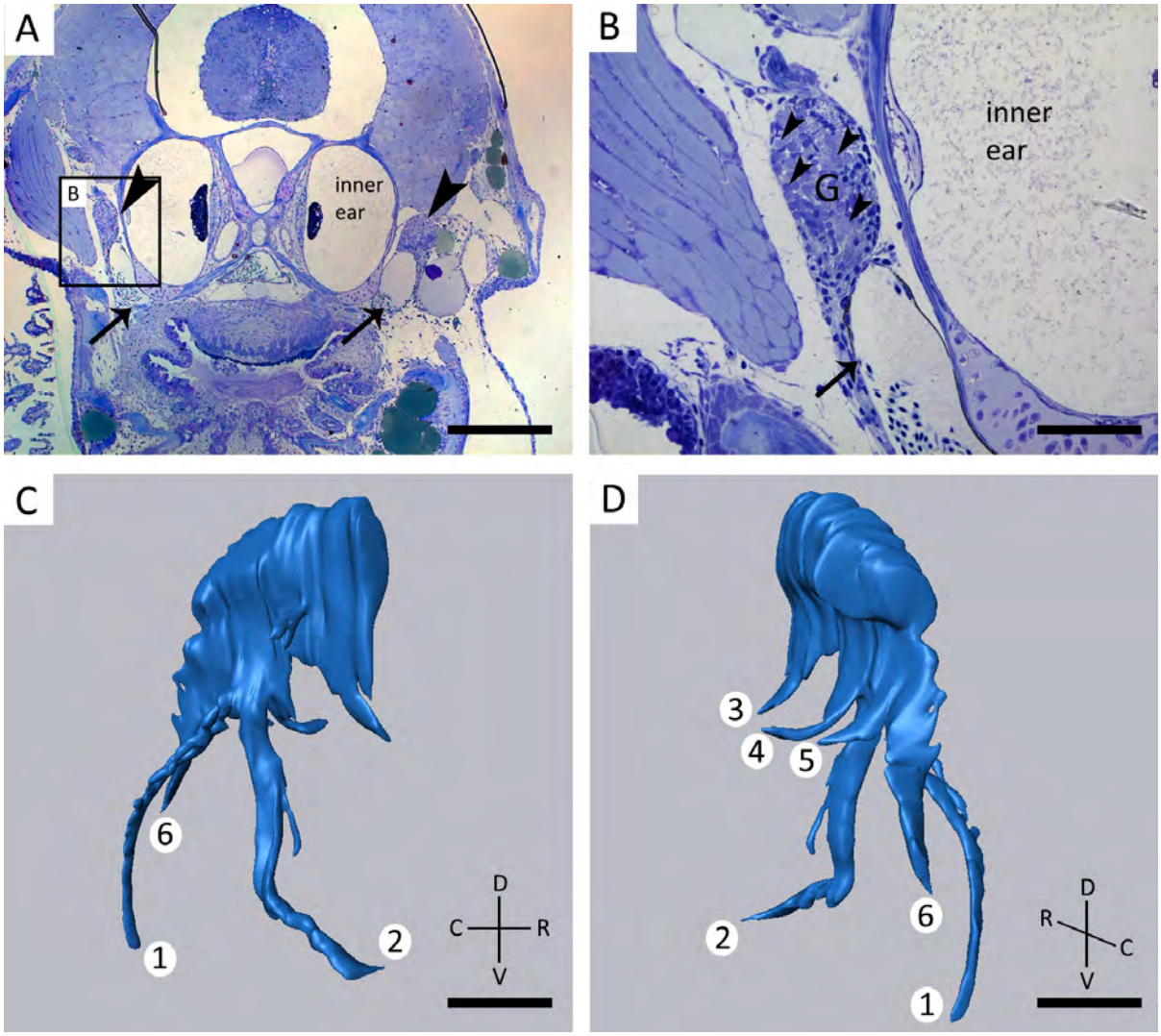


Figure 12: Vagal nerve ganglion and its branches
(A, B) Toluidine blue stained transverse sections of 8.3 mm SL zebrafish showing the presence of two large ganglia (G) (A, arrowheads) dorsal to the developing dentition, lateral to the inner ear, and apposed to the posterior cardinal vein (arrow, A, B). Larger magnification of the area indicated in A, clearly shows the cluster of neuronal cell bodies (B, arrowheads). (C, D) 3D reconstruction of the ganglion shown in A on the right body side. Note the six different branches, two of which (1, 2) have a common origin and course ventrally, three branches run medially (3, 4, 5), and a final branch runs caudally (6). C, caudal; D, dorsal; R, rostral; V, ventral. Scale bars: 200 µm (A), 50 µm (B, C, D).

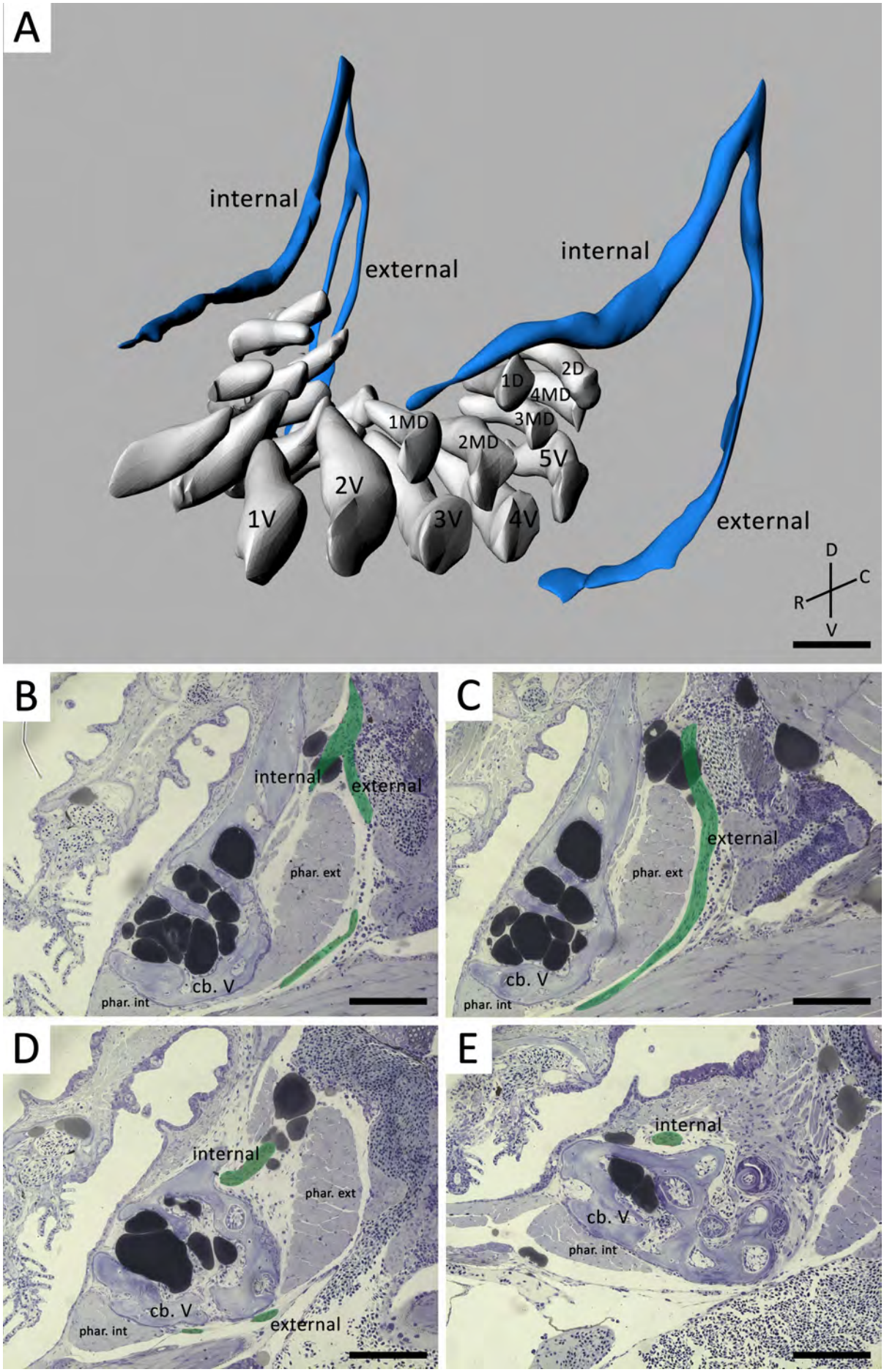
Branches of the vagal nerve innervate the pharyngeal jaws

To study the innervation of the dentition we performed an in-depth light microscopical study of both transverse and sagittal toluidine blue stained sections of 9.5 mm SL zebrafish. Individual nerve bundles could be clearly traced and identified. A 3D reconstruction was made of 188 consecutive semithin (1 µm) sections for visualization and interpretation. Both on the anterior and posterior side of the dentigerous area the nerve branches issuing from the vagal sensory ganglion could be clearly observed and visualised (Figure 13A).

The internal and external nerve branch issue from a common stem connected to the vagal ganglion, as described earlier (Figure 13B). The external branch runs ventrally alongside the branchial musculature positioned posteriorly from the fifth ceratobranchials (Figure 13C). Eventually it bends medially and runs along the ventral side of the ventral tooth row and ceratobranchials (Figure 13D). The second, internal branch courses ventrally over a short distance and soon bends medially towards the teeth. Contrary to the external branch, the internal branch runs along the dorsal side of the ceratobranchials, and passes dorsally to the dorsal tooth row (Figure 13B, E). Surprisingly, in the specimen used for 3D reconstruction, the external branch bifurcated on one side, as opposed to the other side. This could not be observed in other specimens.

Figure 13: Vagal nerve branches innervate the pharyngeal jaws

(A) 3D visualization of a 9.5 mm SL zebrafish dentition demonstrating the main nerve branches (blue) in the vicinity of the developing teeth (white). Note the presence of lipids (black areas) that are revealed through postfixation with OsO_4. The internal and external branches emerge from a common stem on the postero-dorsal side of the dentition. Note that the internal branch is split up in two bundles on one body side. The teeth are organised in a ventral (1V-5V), mediodorsal (1MD-4MD), and dorsal (1D-2D) row. (B-E) Consecutive sagittal toluidine blue stained sections of a wild type zebrafish (SL = 8.3 mm) showing how the internal and external branch (pseudocoloured green) course in relation to both internal and external pharyngoclavicularis muscles (phar. int./ phar. ext.), and the fifth ceratobranchials (cb. V). The external branch extends ventrally and bends in a medial direction along the ventral side of the dentition. The internal branch on the other hand, bends medially almost instantly thus running along the dorsal side of the dorsal tooth row. C, caudal; D, dorsal; R, rostral; V, ventral. Scale bars: 50 µm (A), 100 µm (B-E).


A
internal
external
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1D
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3MD
1MD
2MD
5V
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B
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external
phar. ext
cb. V
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C
external
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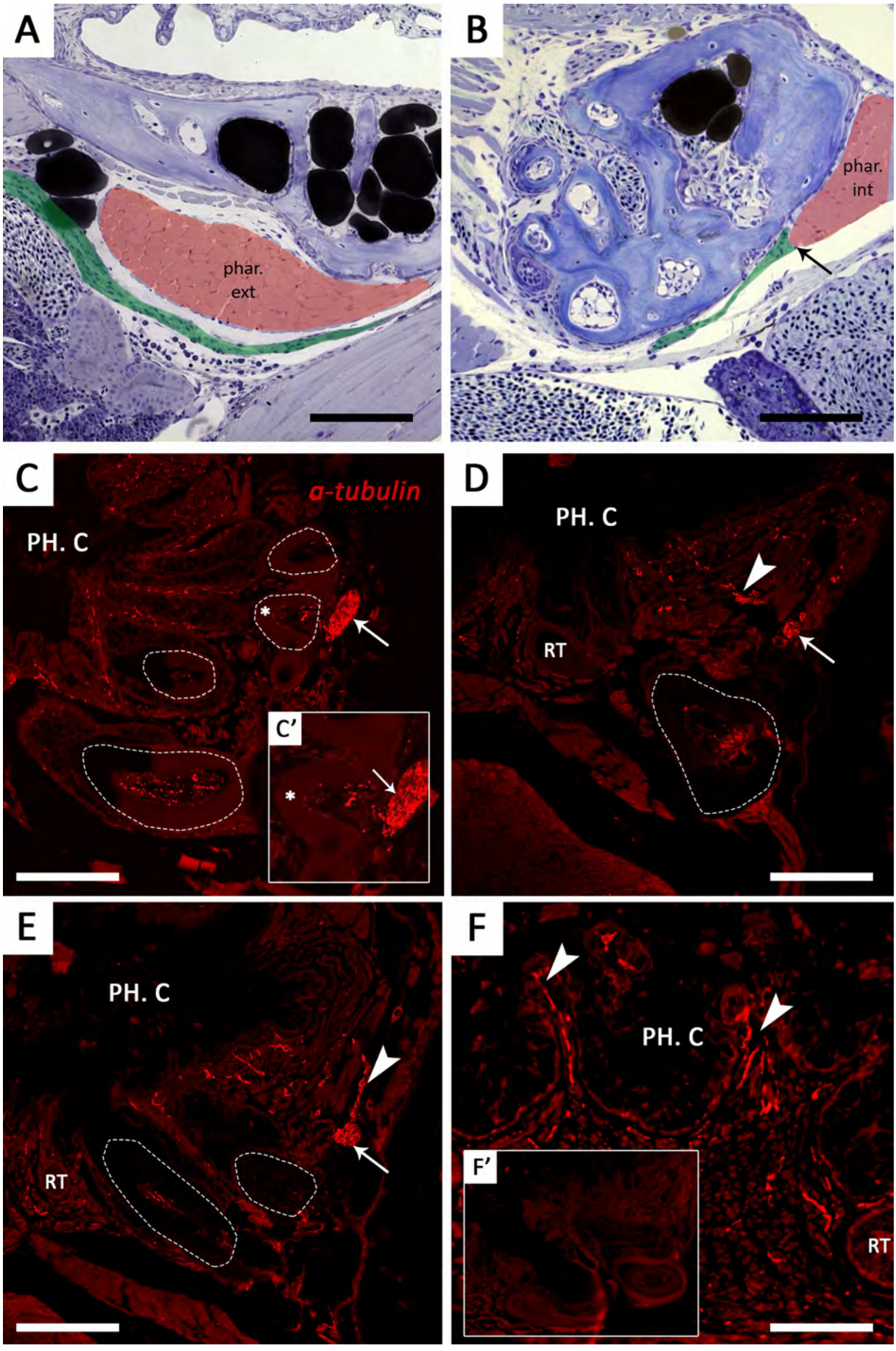
phar. int

We next attempted to identify the targets that are innervated by both the external and internal branch (Figure 14). On sagittal semithin sections, the external branch can be seen running in very close proximity to the pharyngoclavicularis externus muscle, positioned just posteriorly of the fifth ceratobranchials (Figure 14A), and terminates at the pharyngoclavicularis internus muscle on the cranio-ventral side of the dentition (Figure 14B). The internal branch on the other hand, continues running anteriorly and at tooth position 1D, penetrates the bone. The internal branch now runs at the base of the teeth in cranio-ventral direction. Due to a decrease in diameter of the nerve bundle, distinguishing it from the surrounding tissue proved difficult on semithin sections. Hence, immunohistological staining was performed to visualize the nerve bundles. This allowed us to identify the internal branch to be responsible for the pulpal innervation of the teeth. As it passes at the base of the functional teeth, it gives off branches running towards the dental pulp (Figure 14C). Smaller branches can also be seen to ascend towards the pharyngeal epithelium (Figure 14D, E). These smaller branches likely innervate the taste buds located at the crests between adjacent epithelial crypts, and the oral mucosa that surrounds the teeth (Figure 14F).

Figure 14: Targets of internal and external branch
(A, B) Sagittal toluidine blue stained sections of a wild type 9.5 mm SL zebrafish. In both sections anterior is to the right. Note the presence of lipids (black areas) that are revealed through postfixation with OsO_4. The external branch (pseudocoloured green) passes close to the external pharyngoclavicularis muscle (phar. ext, pseudocoloured red) at the posterior side of the dentition (A). The external branch appears to terminate (arrow) at the internal pharyngoclavicularis (phar. int) muscle on the cranioventral side of the dentition (B). (C-F) Immunohistochemical detection of nerve fibres in the dentition of zebrafish. Transverse paraffin sections of adult fish were stained with a primary antibody against acetylated tubulin (α-tubulin). Functional teeth are indicated using dashed lines. (C) The internal branch (arrow) passes at the very base of the functional teeth (dashed line) after having penetrated the ceratobranchial bone at tooth position 1D (not shown). (C') Enlargement of the functional tooth indicated in C (*); smaller branches appear to enter the pulp cavity of the tooth. (D-E) Furthermore, smaller axons (arrowheads) extend from the internal branch (arrow) towards the pharyngeal epithelium, where they probably innervate the oral mucosa and taste buds located between the epithelial crypts (F). (F') Negative control section in which the primary antibody has been omitted. PH. C.: pharyngeal cavity; RT: replacement tooth. Scale bars: 100 µm (A-E), 50 µm (F).


A
phar.
ext
B
phar.
int
C
α-tubulin
PH. C
C'
D
PH. C
RT
E
PH. C
RT
F
PH. C
F'
RT

Innervation of individual teeth

Immunohistological staining revealed nerve fibres in the dental pulp but never in the dentine (Figure 15A). This was confirmed in two different individuals. All functional teeth studied in these two specimens (n = 44), showed specific staining in the pulp. However, we could not detect nerve fibres in replacement teeth, regardless of the stage of differentiation (initiation, morphogenesis, early cytodifferentiation) (Figure 15B). Only at stages of very late cytodifferentiation, i.e. in teeth nearing attachment, could nerve fibres be observed penetrating the pulp cavity at the tooth base (Figure 15C).

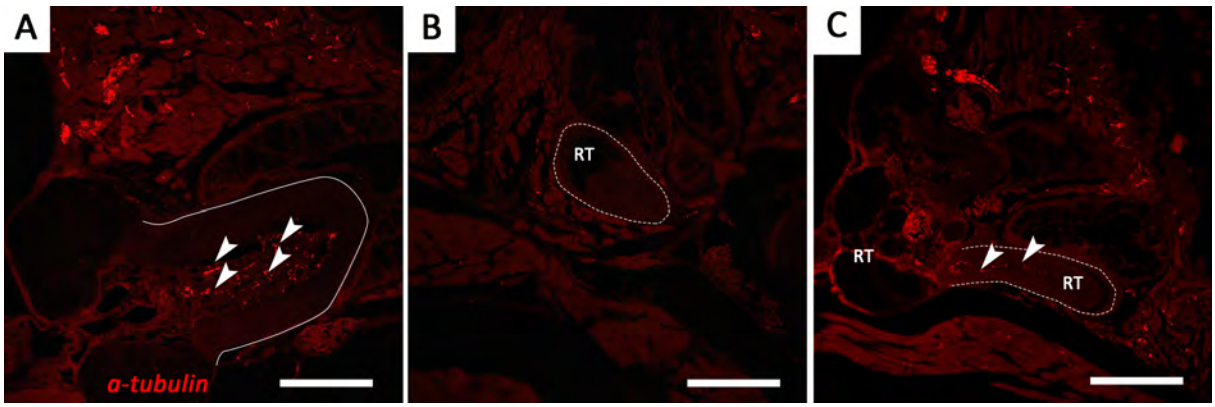


Figure 15: Innervation of individual teeth

Immunohistochemical detection of nerve fibres in both functional teeth (full line) and replacement teeth (dashed line), on transverse paraffin sections of adult zebrafish stained with a primary antibody against acetylated tubulin (α-tubulin). (A) Functional teeth clearly contain many small nerve fibres (arrowheads) in the dental pulp. (B) Replacement teeth (RT), however, are completely devoid of axons at all stages of differentiation. (C) Only at stages of very late cytodifferentiation, i.e. nearing attachment, could nerves (arrowheads) be seen entering the tooth at the base. Scale bars: 50 µm (A), 150 µm (B, C).

2.5 DISCUSSION

This study aimed to improve our knowledge on the nervous supply of the fifth branchial, tooth-bearing, arch (pharyngeal jaw) in zebrafish, and the spatial relationship between nerve bundles and developing teeth. Using both serial semithin sections and immunohistochemistry, we were able to identify the main nerve branches coursing along the tooth-bearing ceratobranchials and the point from where they originate. In addition, we found evidence of nerve fibres penetrating the pulp.

Vagal sensory ganglion and its branches

A large vagal sensory ganglion on each body side gives off branches running towards the dentigerous region. This is in line with our hypothesis that the vagal nerve not only innervates the other, gill-bearing, branchial arches 1-4 (also termed pharyngeal arches 3-6), but also the fifth, tooth-bearing, branchial arch. According to Beaumont and Cassier (1994), a branchial nerve usually consists of a pharyngeal ramus, pre- and post- trematic ramus, and an intestinal ramus. Because the teeth develop on the arch behind the last gill slit ("trema"), the ramus concerned is necessarily a posttrematic ramus. We therefore termed the two branches running towards the teeth the internal and external branch of the posttrematic ramus of the vagal nerve. The three nerve bundles running medially are potentially the pharyngeal branches, contradicting Beaumont and Cassier (1994) who only describes one pharyngeal branch. Finally, the bundle running caudally from the ganglion is probably the intestinal ramus responsible for the innervation of the gut, as described by several authors (Higashijima et al., 2000, Holzschuh et al., 2005, Jonz and Zaccone, 2009).

Branches of the vagal nerve innervate the pharyngeal jaws

Based on our light microscopical study and 3D reconstructions, and on published data (Higashijima et al., 2000, Holzschuh et al., 2005, Jonz and Zaccone, 2009) we have identified the vagal nerve as responsible for the innervation of both the pharyngeal jaws and teeth (summarized in Figure 16).

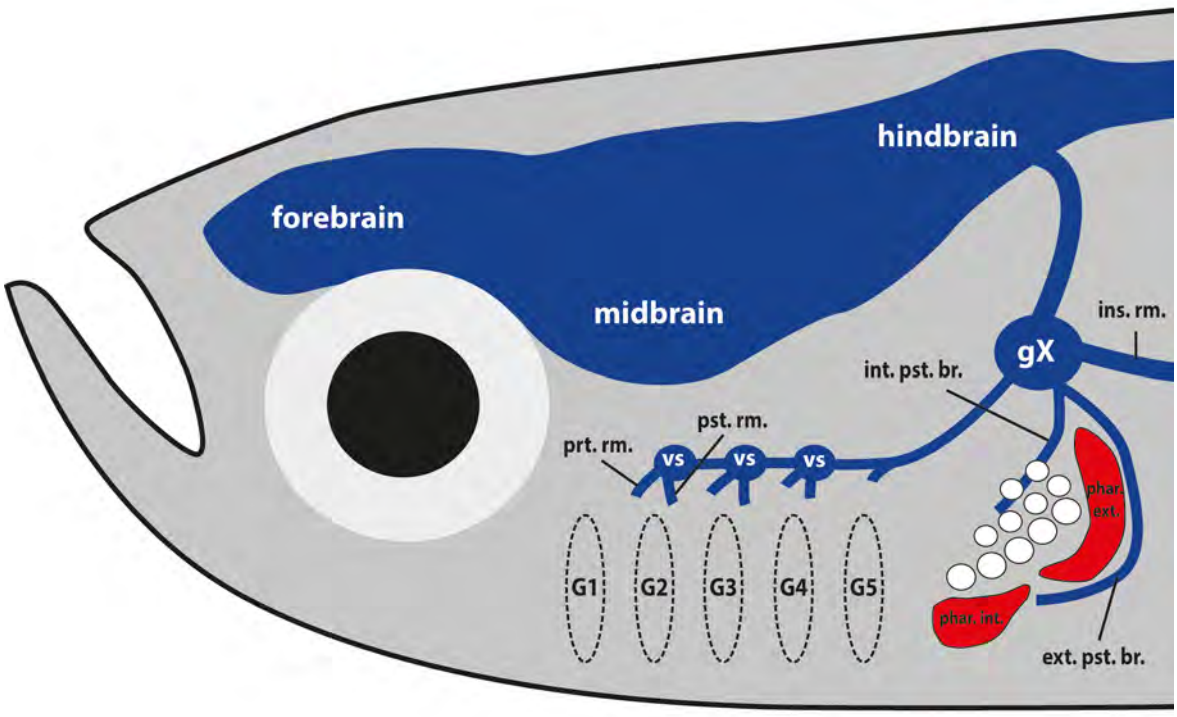


Figure 16: Schematic drawing of innervation to teeth in zebrafish
Schematic representation of the main nerve branches in the vicinity of the dentition. The internal branch of the posttrematic ramus (int. pst. br.) of the vagal nerve is responsible for the innervation of the teeth along with the taste buds. The external branch of the posttrematic ramus (ext. pst. br.) on the other hand innervates the branchial musculature, i.e; the internal and external pharyngoclavicularis (phar. int./ phar. ext.). Both internal and external branches emerge from a large vagal ganglion (gX), which also gives rise to the intestinal ramus (ins. rm.). Also note the smaller vagal sensory ganglia (vs) positioned dorsally of gill slits two to four (G2-G4) which give rise to both pretrematic (prt. rm.) and posttrematic (pst. rm.) rami. The first gill slit (G1) is innervated by branches of the glossopharyngeal nerve (nIX) (not shown).

The fifth ceratobranchials in zebrafish appear to be innervated solely by a posttrematic ramus of the vagal nerve, issuing from a large sensory vagal ganglion. Several authors report the presence of not just the large sensory ganglion but also of several smaller sensory ganglia at the more anteriorly positioned gill arches giving off pretrematic and posttrematic rami as well (Higashijima et al., 2000, Holzschuh et al., 2005, Jonz and Zaccone, 2009). However, contrary to what we expected, the posttrematic ramus on either body side is divided in both an internal and external branch. The bifurcation in the external branch on one side, as opposed to the other body side, was not observed in other specimens and could therefore be an artefact of the 3D rendering. Since the external branch appears not to be involved in the innervation of the teeth, but rather represents the motoric innervation of the branchiomeric musculature, the potential asymmetry is not relevant in the light of discussing the innervation of zebrafish teeth.

The internal branch of the posttrematic ramus probably transmits sensory innervation from the teeth, taste buds, and oral mucosa. A similar innervation pattern has already been observed in two other teleosts, the perch (*Perca fluviatilis*) and the rainbow trout (*Oncorhynchus mykiss*) (Dunelerb et al., 1993), where taste buds are said to be innervated by both post- and pretrematic rami of the vagal nerve. The external branch of the posttrematic ramus on the other hand likely transmits motor information to the branchiomeric musculature. This is also the case for several species of Tetraodontiformes, where posttrematic rami of the vagal nerve are involved in the innervation of the internal and external pharyngoclavicularis muscles (Nakae and Sasaki, 2008).

Both internal and external branches of the posttrematic ramus emerge directly from their dorsal ganglion. Therefore, one could assume these nerves to contain solely viscero-afferent fibres, since somato-efferent fibres have been described in zebrafish to emerge directly from nuclei in the central nervous system and to course directly towards their targets, i.e. branchiomeric musculature (Beaumont and Cassier, 1994, Chandrasekhar et al., 1997). However, given the possible involvement of the external branch of the posttrematic ramus in delivering motor information to both the internal and external pharyngoclavicularis, we assume these posttrematic branches not only to transmit sensory information, but also motor signals. In humans, motor fibres of the trigeminal nerve likewise pass through the trigeminal ganglion without having their cell bodies in the ganglion; rather they are located in nuclei in the central nervous system (Nilsson, 1983).

The combination of motor and sensory nerve fibres has also been described in the light of vasomotoric control of the vasculature in the gills (Nilsson, 1983, Beaumont and Cassier,

1994, Jonz and Zaccone, 2009). In mammals, axons present in the pulp take part in the regulation of pulpal blood flow (Kim, 1990, Kerezoudis et al., 1992, Olgart, 1996, Pagella et al., 2014). Given a possible role of motor fibres in regulating blood flow, it is important to note that the dentigerous area in zebrafish is richly supplied with blood (Crucke and Huysseune, 2013). We have recently raised the hypothesis that blood vessels act instructively to initiate tooth development in zebrafish (Crucke and Huysseune, 2013). However, in a follow-up study, we demonstrated that blood vessels are not required for tooth initiation but rather allow the teeth to further grow and develop (Crucke and Huysseune, 2015). Nerves in the dentigerous region could be involved in influencing the vascular elements present through secretion of so-called angioneurins, i.e. signalling molecules affecting both neural and vascular functions (Zacchigna et al., 2008a). Moreover, it has been shown in the skin of mice that peripheral nerves provide a template for developing arteries to guide their growth and development toward their target (Mukouyama et al., 2002). This cross-talk between vascular and neural networks is termed *the neurovascular link* (Carmeliet, 2003b, Zacchigna et al., 2008b, Ulrich et al., 2011). To which degree the nervous system influences the vascular network and vice versa during the process of tooth development, still needs to be determined.

Innervation of individual teeth

Apart from studies performed on the cichlid *Tilapia mariae* (Holje et al., 1986, Tuisku and Hildebrand, 1994, Tuisku and Hildebrand, 1996), little information is available on innervation of teleost teeth. Here, we have, for the first time, clearly demonstrated the presence of nerve fibres in the pulp cavity of functional teeth in zebrafish. While this may be expected based on a comparative basis (e.g. data from mouse, rat, human) (Zmijewska et al., 2003, Luukko et al., 2005a, Luukko et al., 2008), it may be somewhat surprising considering the continuous turnover of the teeth and their short functional life time. Therefore, nerves are probably more involved in concepts such as vasomotoric control rather than pain sensation (teeth are replaced anyway). The nerve fibres in the pulp of zebrafish issue from an internal branch of the posttrematic ramus of the vagal nerve. In *T. mariae* and mammals, on the other hand, dental nerves originate from the trigeminal nerve (Holje et al., 1986) and the inferior/superior alveolar nerve (Hildebrand et al., 1995) respectively. Given the location of zebrafish teeth deep within the oral cavity, a difference in innervation when compared to oral teeth is to be expected. Furthermore, different from what we know for mice and humans, the dental nerves in zebrafish appear to be limited to the pulp cavity, whereas in mammals, nerve fibres penetrate into the dentine as well (Fearnhead, 1957, Byers, 1980).

Interestingly, we did not find any evidence for nerve fibres in developing replacement teeth. All stages of differentiation were devoid of nerve fibres. Only at very late stages of cytodifferentiation, i.e. nearing attachment, did we see nerves entering at the base of the replacement tooth. At this stage, teeth become functional allowing nerves to serve their function for the tooth. Likewise, blood vessels could only be seen penetrating at the base of replacement teeth at very late stages of differentiation (Crucke and Huysseune, 2013), further suggesting a developmental and/or functional link between both. Moreover, the initiation of a replacement tooth (i.e. upon attachment of the predecessor) might then be potentially achieved through the secretion of certain growth factors by sensory neurons that in turn could activate mesenchymal stem cells, e.g. periarterial cells as was shown for the neurovascular bundle present in the mouse incisor (Zhao et al., 2014). In mice, nerves enter the dental pulp at late bell stage, i.e. after the onset of enamel formation (Moe et al., 2008).

The late penetration of nerves into the dental pulp, both in mice and zebrafish, suggests a relative independence between the establishment of the innervation on the one hand, and the process of tooth development on the other hand. Yet, prior to the penetration of nerves in the dental pulp, growth of the dental trigeminal axons in mice occurs concomitantly with advancing tooth formation (Hildebrand et al., 1995). This coordinated development between nerves and teeth hints towards a possible neuronal influence on tooth development as was already suggested by Pearson (1977). Nonetheless, only two studies have yet been able to show a direct influence of nerves on the processes of tooth development and replacement. In *T. mariae*, unilateral denervation of the trigeminal nerve ceased tooth turnover on the operated side (Tuisku and Hildebrand, 1994). Removing inferior alveolar neurovascular structures in puppies with erupted deciduous teeth stopped the eruption of permanent teeth (Harputluoglu, 1990, Tuisku and Hildebrand, 1994). These two studies suggest an instructive role of dental nerves in tooth development and replacement. The arrest in tooth development in these studies might have succeeded through removing the potential source of mesenchymal stem cells (e.g. nerve-associated glia), or in removing the inductive signal produced by sensory nerves to induce the differentiation of periarterial cells as was demonstrated in the mouse incisor (Zhao et al., 2014).

2.6 CONCLUDING REMARKS

In conclusion, having established the essential morphological background information on innervation of zebrafish teeth, we can now investigate whether or not dental nerves in zebrafish fulfil an instructive or rather permissive role during odontogenesis. It is clear that apart from investigating direct effects on tooth development, future research should also focus on mutual interactions of the nerves with the vascular system (i.e., the neurovascular link).

2.7 ACKNOWLEDGEMENTS

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Chapter 3

Blocking VEGF signalling delays development of replacement teeth in zebrafish

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Key words: Dentition; tooth replacement; SU5416; VEGFR-2; blood supply; *Danio rerio*

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3.1 ABSTRACT

The dentition in zebrafish is extremely richly vascularized, but the function of the vasculature, in view of the continuous replacement of the teeth, remains elusive. Through application of SU5416, a vascular endothelial growth factor receptor inhibitor, we studied the role of the blood vessels in the dentition of the zebrafish. Blocking angiogenesis through this inhibitor did not affect the development of first-generation teeth nor the first tooth replacement. In juvenile specimens, such inhibition produced a delay in the developmental state of the replacement tooth compared to what was expected based on the maturation state of the functional tooth. Furthermore, we observed a difference in the distance between blood vessels and developing replacement teeth between treated and non-treated fish. In conclusion, our results provide support for a nutritive, rather than an inductive, function of the vasculature in the process of tooth development and replacement.

3.2 INTRODUCTION

Over the past few decades, there has been an enormous progress of knowledge on the genetic and molecular mechanisms that regulate tooth development, not just in classical models such as the mouse (Hu et al., 2006; Yen and Sharpe, 2008), but also in non-mammalian species (Jackman et al., 2013; Wise and Stock, 2010). However, certain aspects have remained rather poorly studied. One of them is the role of vascularization during tooth development and replacement.

Studies focussing on the vascular supply of teeth are mostly limited to mammals and concern the intrinsic blood supply via the pulp cavity (Boyer and Neptune, 1962; Kindlova and Matena, 1962). One line of recent studies focuses on perivascular cells such as pericytes, and their potential to act as a source of mesenchymal stem cells (Crisan et al., 2012).

Here, we aim at studying the role of blood vessels in the development and replacement of (first-generation) teeth in a non-mammalian model, the zebrafish (*Danio rerio*). The zebrafish is a small teleost fish belonging to the cyprinids, and is widely used as a vertebrate model organism for genetic, molecular, and developmental research (Lele and Krone, 1996; Roush, 1996). Similar to most other tooth-bearing non-mammalian vertebrates, zebrafish replaces its teeth throughout life. The zebrafish has no oral dentition; its teeth are restricted to the pharyngeal region and are implanted on the fifth branchial arch (Huysseune et al., 1998; Van der heyden et al., 2000).

Earlier we have shown that the dentigerous area in zebrafish is richly vascularized (Crucke and Huysseune, 2013). The teeth are surrounded by a sinusoidal cavity originating from the hypobranchial artery. Furthermore, we have demonstrated that the pulpal cavity of first-generation teeth in zebrafish is completely devoid of vascular elements. In addition, replacement teeth only become vascularized relatively late in their development, i.e. during late cytodifferentiation stage (Crucke and Huysseune, 2013).

Given the extremely rich vascularization, and in particular the presence of a large sinusoidal cavity encircling the teeth, the question rises as to what is the role of these vessels in the continuous replacement of the teeth. Here, we address this question by preventing new blood vessel formation through interfering with VEGF signalling. In particular, we use SU5416, an anti-angiogenic compound widely employed in both developmental and clinical research (Fiedler et al., 2003; Kuenen et al., 2002; Serbedzija et al., 1999), to study the role of the vasculature both in initiation of first-generation teeth, their replacement, as well as in ongoing tooth replacement in juveniles.

3.3 Material and Methods

Animal husbandry

Wild-type zebrafish and Tg(fli1:EGFP) were obtained from ZIRC (University of Oregon) and kept in a 14 h/ 10 h light/dark cycle at 28.5 °C (Westerfield, 1993). Embryos, larvae and juvenile fish were raised in egg water and sacrificed according to the Belgian law on the protection of laboratory animals (KB d.d. 13 September 2004) by an overdose of MS222 (3-aminobenzoic acid ethyl ester). Embryos/larvae 40–115 hours post-fertilization (hpf), and one month old juvenile zebrafish (standard length, SL, between 6.8 and 10.2 mm) were used.

Alkaline phosphatase staining

Blood vessels in larval zebrafish possess strong endogenous alkaline phosphatase activity, allowing an easy and rapid visualization of the vasculature in larval zebrafish (Kamei et al., 2004). Larvae were fixed in 4 % paraformaldehyde in phosphate buffered saline - tween (PBS-T) for 30 – 60 minutes and stained for alkaline phosphatase activity according to the protocol described in Kamei et al. (2004).

VEGFR inhibition

We used SU5416 (Sigma-Aldrich, Bornem, Belgium), a potent and selective inhibitor that blocks the VEGF-dependent kinase activity associated with the Flk-1/KDR receptor (Fong et al., 1999). This compound has already been shown to prevent blood vessel formation in zebrafish (Serbedzija et al., 1999). The inhibitor was dissolved in dimethylsulfoxide (DMSO), and a stock solution of 10 mM was prepared. This stock solution was directly added to the embryo rearing medium to achieve a final concentration of 1 µM. Fifty percent of the medium was exchanged on a daily basis. Thirty embryos aged 40 hpf and 40 juvenile specimens between 6.8 and 10.2 mm SL were treated for variable periods. To study the effect on the development of the first-generation teeth ($3V^1$, $4V^1$, $5V^1$) along with the development of the first replacement tooth ($4V^2$), treatment was started at 40 hpf and lasted until 115 hpf. To check the effect on tooth replacement in a fully developed, cycling dentition, we treated one month old juveniles for ten days. Negative control specimens were incubated using 0.1% DMSO. As a positive control we checked whether or not certain blood vessels developed in the treated fish when compared to the control fish (Figure 17). All experiments were authorized by the local ethical committee on laboratory animal experimentation (EC2011-037).

Tissue processing

Larvae and juvenile fish were fixed in a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde buffered with 0.2M cacodylate (pH 7.4) for 2 hours at room temperature. Juvenile zebrafish were decalcified by adding 0.1M EDTA to the fixative solution for one to several weeks at 4°C. The decalcifying solution was refreshed every two days. After fixation, animals were rinsed in 0.2M cacodylate buffer containing 10% sucrose, and postfixed for two hours at room temperature with 1% OsO_4 in 0.2M cacodylate buffer containing 8% sucrose. After rinsing in the same buffer, specimens were dehydrated through a graded series of ethanol and embedded in epon. Serial semithin (1 μm) sections were made using a Reichert-Jung ultra-cut ultramicrotome (Leica, Vienna, Austria). These sections were stained using toluidine blue, and mounted in DePeX (Gurr, BDH laboratory, UK). All sections were examined using a Zeiss Axio Imager Microscope and photographed using an Axiocam MRC videocamera. Reconstructions were made using the software program Amira® (v5.3.3.). Finally, 3D models were photographed from different perspectives using Rhinoceros® (v5.0).

Statistical analysis

To quantify the distance between developing replacement teeth and the surrounding sinusoidal cavity, all tooth positions were examined and the distance was measured using the free image-processing program ImageJ. Data was compared using the mean with its respective standard deviation to quantify variability, and statistically significant differences were determined using Student's *t*-test. Results were considered significant when compared to control group if *p*-value < 0.05 (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4 Results

A concentration of 1 µM effectively inhibited new blood vessels from developing during the period of treatment, both in larval and in one month old fish. This was confirmed using both Tg(fli1:EGFP), and alkaline phosphatase staining in WT fish (Figure 17) (Serbedzija et al., 1999).

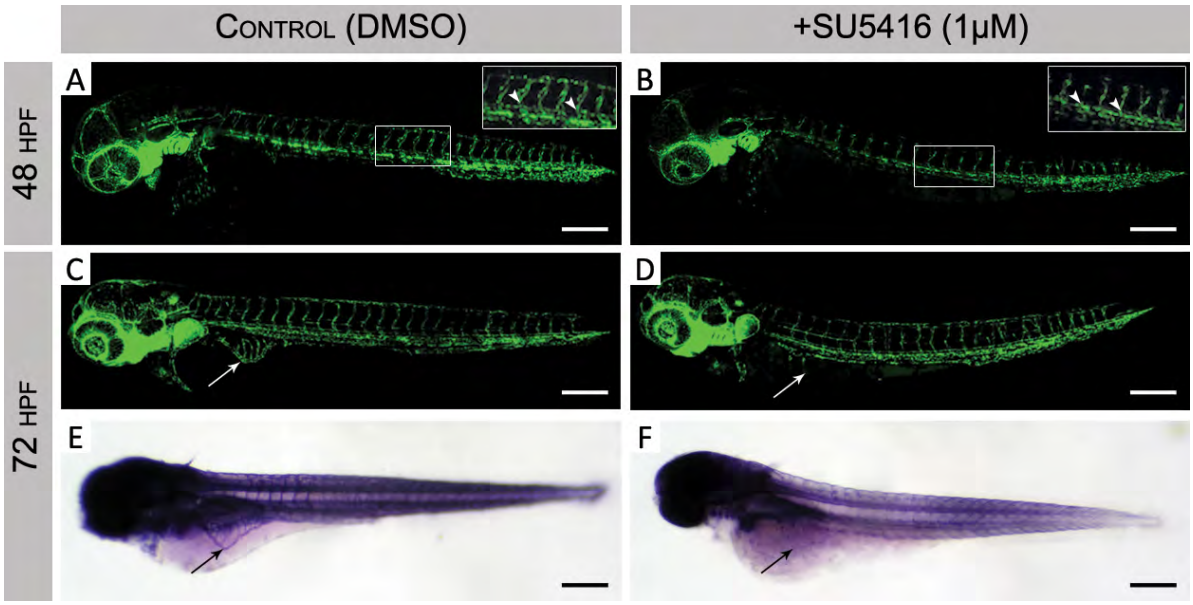

Figure 17: Effects of SU5416 on vascular development
Comparison of the vasculature between control (=DMSO-treated) (n = 3) and SU5416-treated (n = 3) animals. Upper panels (A, B) show 48 hpf *Tg(fli1:EGFP)* fish; lower panels show 72 hpf of both *Tg(fli1:EGFP)* (C, D) and alkaline phosphatase stained WT fish (E, F). Both upper and lower panels clearly show the arrest in the developing vasculature due to the addition of the compound SU5416 at 20 hpf (A, B), and at 48 hpf (C, D, E, F). Insets represent larger magnification of areas indicated in A and B respectively. At 48 hpf the intersegmental vessels (arrowheads, A) have clearly formed, while this is not the case for the treated fish in which the intersegmental vessels (arrowheads, B) have failed to fully develop. Also, at 72 hpf the subintestinal vessels are clearly visible (arrow, C, E), whereas in the treated fish they are completely lacking (arrow, D, F). This was confirmed using both transgenic fish and alkaline phosphatase staining in WT fish. Scale bars are 50 µm.

VEGFR inhibition does not affect tooth initiation and first tooth replacement

In zebrafish, the first tooth ($4V^1$) already starts to develop at 48 hpf. At around 80 hpf this tooth becomes attached, at which time the first replacement tooth is initiated. Therefore, we started the treatment at 40 hpf. Treatment continued until 115 hpf.

At 115 hpf, not only $4V^1$, but also $3V^1$ and $5V^1$ are present in the dentition of the control fish (n = 3), in addition to the first replacement tooth ($4V^2$). Tooth $4V^1$ has already attached, while

both $3V^1$ and $5V^1$ are in late cytodifferentiation stage. Furthermore, the first replacement tooth, $4V^2$, has reached early cytodifferentiation stage (Figure 18).

Treated fish (n = 3) display a similar condition at 115 hpf. All three first-generation teeth ($3V^1$, $4V^1$, $5V^1$) are present and have reached the same developmental stage as compared to the control fish. The first replacement tooth ($4V^2$) has also started its development and has reached early cytodifferentiation stage. Hence, there is no difference between control and treated fish in terms of presence and developmental stage of the different teeth. This has been confirmed in six serially sectioned specimens.

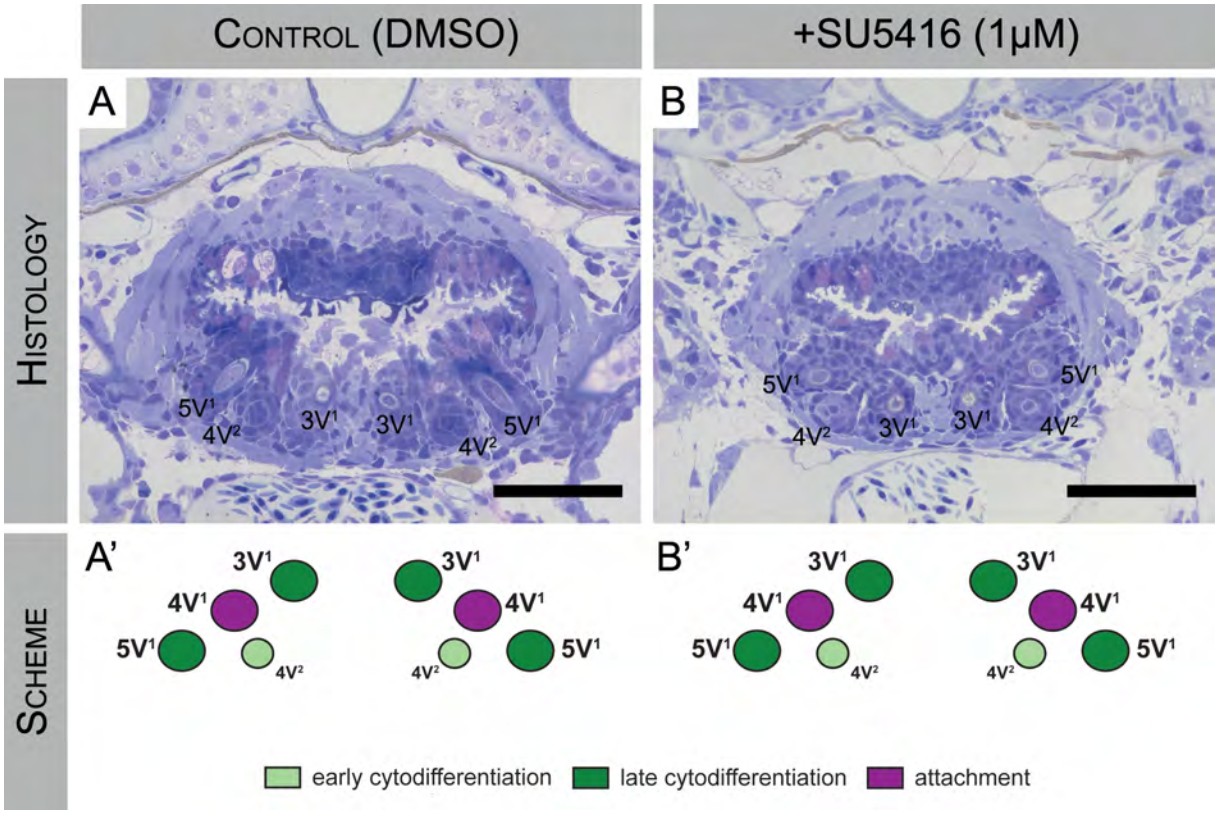

Figure 18: Inhibition of VEGFR does not influence first-generation teeth
Transverse toluidine blue stained sections of 115 hpf old larvae (A, B). No difference is observed in developmental stage of the teeth between the control (=DMSO-treated) (n = 3) (A) and the SU5416-treated (n = 3) (B) fish. In both situations al three first-generation teeth are present along with the first replacement tooth, $4V^2$. Note that $4V^1$ has an orientation that is slightly different from $3V^1$ and $5V^1$ and is therefore not visible in this section. The corresponding scheme (A', B') shows the developmental stage of the teeth present in the dentition at 115 hpf. Scale bars are 50 µm.

VEGFR inhibition delays the development of replacement teeth

To determine the role of the vasculature in the continuously renewing dentition, we treated wild type zebrafish using 1 μM SU5416 for a period of ten days. Each tooth position was next scored on serial semithin sections for the maturation state of the functional tooth along with the developmental state of its associated successor. Each functional, erupted tooth was assigned to one of five different categories based on its state of functionality, as assessed by the presence and the state of the differentiated cells in the pulp cavity: attaching, young, mature, old, and absent. Its associated replacement tooth was assigned to one of the following four categories based on the state of differentiation: successional lamina (sl), morphogenesis (M), early cytodifferentiation (EC), and late cytodifferentiation (LC) stage. Observations were performed double-blind on 211 tooth positions in ten serially sectioned specimens (Table 1)

	Ventral		Mediodorsal		Dorsal		Total		Percentage	
Teeth	CTR	TRT	CTR	TRT	CTR	TRT	CTR	TRT	CTR	TRT
Attaching										
sl	1	3	1	0	0	0	2	3	100.0	100.0
M	0	0	0	0	0	0	0	0	0.0	0.0
EC	0	0	0	0	0	0	0	0	0.0	0.0
LC	0	0	0	0	0	0	0	0	0.0	0.0
Young										
sl	5	4	2	2	0	0	7	6	70.0	85.7
M	0	0	1	1	1	0	2	1	20.0	14.3
EC	0	0	1	0	0	0	1	0	10.0	0.0
LC	0	0	0	0	0	0	0	0	0.0	0.0
Mature										
sl	0	10	0	7	0	3	0	20	0.0	39.2
M	0	0	0	3	0	4	0	7	0.0	13.7
EC	5	6	0	3	1	2	6	11	46.2	21.6
LC	5	4	2	6	0	3	7	13	53.8	25.5
Old										
sl	0	7	0	6	0	4	0	17	0.0	19.3
M	0	2	0	1	0	2	0	5	0.0	5.7
EC	1	3	0	0	0	1	1	4	11.1	4.5
LC	3	35	2	16	3	11	8	62	88.9	70.5
Absent										
sl	0	0	0	0	0	0	0	0	0.0	0.0
M	0	0	0	0	0	0	0	0	0.0	0.0
EC	0	0	0	0	0	0	0	0	0.0	0.0
LC	1	5	3	14	3	2	7	21	100.0	100.0
Total	21	79	12	59	8	32	41	170		

Table 1: Relationship between the stage of maturation of the functional teeth and the developmental stage of the replacement tooth in control and treated fish (+SU5416)

Numbers refer to number of tooth loci. Each functional tooth (n = 41 for control [CTR] fish, n = 170 for treated [TRT] fish) was assigned to 1 of 5 categories of maturation (attaching, young, mature, old, and absent) and scored for the developmental stage of its associated replacement tooth: successional lamina (sl), morphogenesis (M), early cytodifferentiation (EC), late cytodifferentiation (LC). Teeth belonging to the 3 tooth rows—ventral, mediodorsal, dorsal—were scored separately. Percentages are given for each combination of functional and replacement teeth.

In all studied specimens, whether control or treated, functional, attached teeth are associated with either a successional lamina or a developing replacement tooth. In general, older functional teeth (mature/old) are associated with replacement teeth in a more advanced state of differentiation (early/late cytodifferentiation). In the control (=DMSO-treated) fish (Left side, Figure 19), 100% of the tooth positions occupied by mature and old functional teeth posses a successor in early/late cytodifferentiation stage, while 90% of all young functional teeth have a replacement tooth that has reached only successional lamina or morphogenesis stage (Table 1). In the SU5416-treated fish (Right side, Figure 19), however, there is a remarkable shift in this relation when compared to the control group. Here, the presence of an older functional tooth does not necessarily imply the presence of an advanced replacement tooth (Figure 19). Indeed, in the treated fish, only 47.1% of the mature functional teeth were associated with a successor in early/ late cytodifferentiation, compared to 100% in the control group (Graph, Figure 19). In addition, in all treated fish, all different tooth rows (ventral, mediodorsal, dorsal) were affected. Furthermore, tooth positions were not always similarly affected. Thus, within the same individual, positions with a functional tooth in the same stage of maturation could display a successor in a different stage of development. This was the case for 23 tooth positions. Finally, and importantly, we did not see any difference in the vascularisation of advanced replacement teeth: both in control and in treated fish, only replacement teeth from late cytodifferentiation stage onwards were vascularized.
In summary, the data indicate that VEGFR inhibition delays the development of a replacement tooth with respect to its predecessor.

Delayed replacement teeth show an increased distance to the surrounding blood vessels

Treated fish displayed a difference in the distance between the sinusoidal cavity surrounding the functional, erupted teeth, and a developing replacement tooth (Figure 20). An erupted tooth possesses a vascularized pulp cavity, while its associated replacement tooth develops in close proximity to the surrounding vasculature. This was the case for all tooth positions (100%) studied in the control fish and was independent of the developmental stage of the replacement tooth. The mean distance between the developing replacement tooth and the sinusoidal cavity surrounding the functional predecessor was 12.16±4.13 µm (n = 32). For replacement teeth of the treated fish that did not show a developmental delay (i.e. expected differentiation stage of replacement tooth based on the maturation state of the functional tooth), the mean distance was 10.7±5.05 µm (n = 111). However, in delayed replacement teeth, the mean distance was 39.4±14.59 µm (n = 48), representing a significant difference (*p*

<0.001, student's *t*-test) when compared to both the control fish and the non-delayed teeth of the treated group.

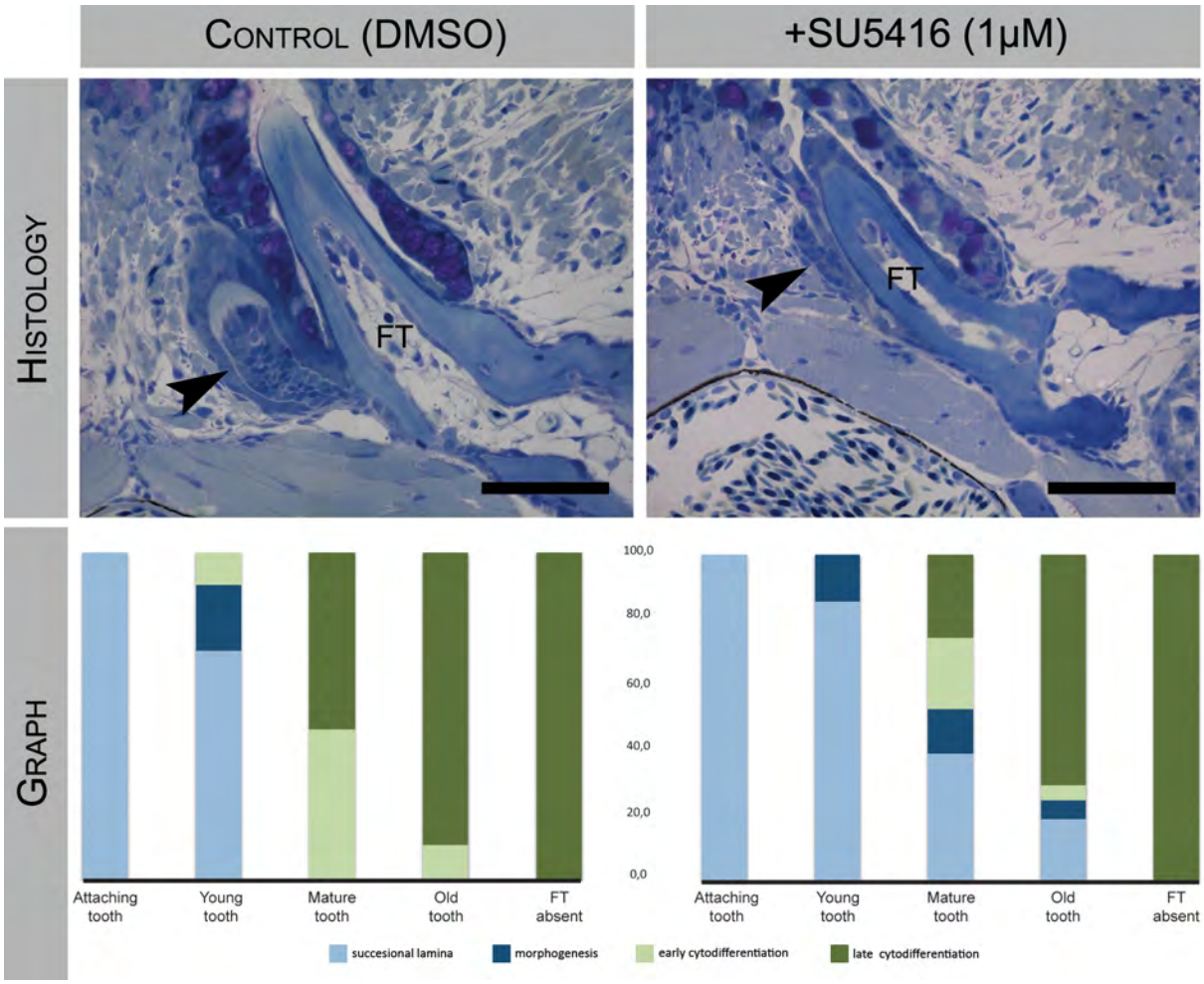

Figure 19: VEGFR treatment delays development of replacement teeth
Transverse toluidine blue stained sections of juvenile zebrafish showing the difference in developmental stage of the replacement teeth between treated (right panels) and control (=DMSO-treated) (left panels) fish. Both panels show an erupted, functional tooth (FT) at a similar stage of maturation. Nonetheless, the associated replacement tooth (arrowhead) differs in developmental stage. In control fish (left panel), the mature tooth is associated with a replacement tooth in late cytodifferentiation stage; in the treated fish (right panel), the mature functional tooth is associated with a replacement tooth that has barely initiated (successional lamina stage). The graphs summarize the data represented in the Table (see Table). Note a clear shift in the relationship between FT and RT in the treated fish, where a replacement tooth in SL or M stage can be found associated with any stage of maturation of the functional tooth, while this is not the case in the control fish.

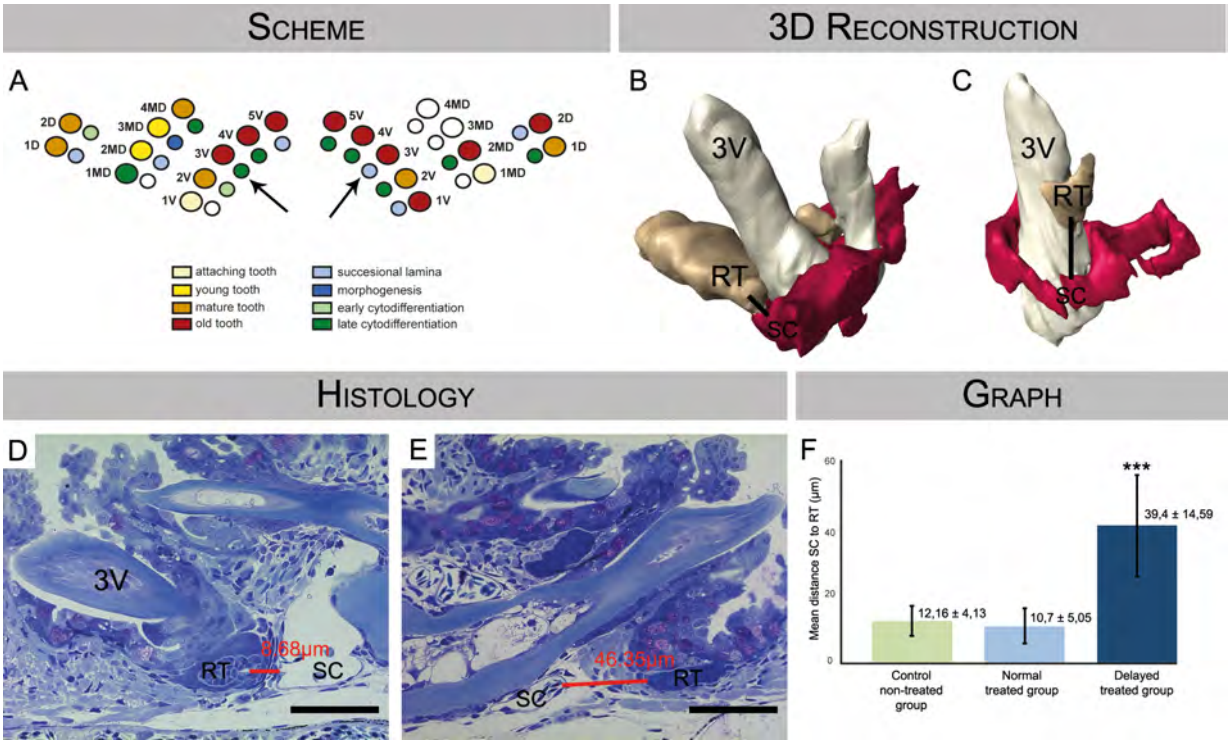

Figure 20: Larger distance to surrounding blood vessels in delayed replacement teeth
(A) Schematic representation of the dentition of a SU5416-treated fish, with indication of all three rows of functional teeth (large circles) and their successors (small circles) on both body sides (1V-5V, 1MD-4M and 1D-2D: teeth of the ventral, mediodorsal and dorsal tooth row, resp.). Arrows indicate two old functional teeth with their associated successor each in a different stage of differentiation. (B, C) 3D reconstructions showing the functional tooth (white) at position 3V (as indicated in the scheme, left in A, right in B) and its corresponding successor (brown, RT) along with the surrounding sinusoidal cavity (red, SC). The black lines indicate the distance between the base of the replacement tooth and the closest point of the sinusoidal cavity. The same two teeth are shown in transverse toluidine blue stained section (D and E, resp.). The red line marks the distance between the replacement tooth (RT) and the surrounding vasculature (SC). Scale bars are 50 µm. (F) The graph shows a comparison between the SU5416-treated group and the control in terms of mean distance between the sinusoidal cavity and the base of the replacement tooth. For the treated group, a distinction is made between teeth showing a developmental delay (Delayed treated group), and those that do not (Normal treated group). The mean distance in the former is significantly different from that in the latter and in the non-treated group (asterisk indicates significance at the p <0.001 level). Data and error bars represent means±SD.

3.5 DISCUSSION

The fact that the dentigerous area in zebrafish is so richly supplied with blood, and, in particular, the presence of a large sinusoidal cavity that surrounds the bases of the teeth (Crucke and Huysseune, 2013) strongly suggests that the vascular system has an important role in the development and/or maintenance of the dentition. Here, we have tried to elucidate the function of the vasculature in the vicinity of the teeth by blocking VEGF signalling.

We were unable to show an effect on initiation of tooth development at early stages of zebrafish development. Given the reported inhibitory effect of 1 µM SU5416 on the formation of blood vessels in embryonic/larval zebrafish (Cross and Claesson-Welsh, 2001, Parng et al., 2002), confirmed also in our controls, we can safely conclude that until 115 hpf, the first-generation teeth develop independently from the vasculature. This corresponds with the observation that the branch of the hypobranchial artery that supplies the dentition only reaches the dentigerous area at 6 days post-fertilization (Crucke and Huysseune, 2013). This is already an indication that the first-generation teeth probably develop independently from the vasculature.

This relative independence from the vasculature for the development of teeth *per se* is confirmed in juvenile stages. Inhibiting the formation of new vessels does not prevent the formation of replacement teeth. However, different from the first replacement tooth, $4V^2$, replacement teeth that develop at a later stage show a clear developmental delay. Yet, there appears to be variation, even within a single specimen, with functional teeth displaying a replacement tooth that is either delayed or not. A possible explanation can be that the non-delayed tooth was already initiated prior to the start of treatment. Alternatively, such a difference could be explained if we assume the initial stages of tooth development (initiation, morphogenesis) to be short living, while the later stages (early/late cytodifferentiation) persist for a longer period. This is not unlikely given that the initial stages of odontogenesis require the mere folding of different cell layers, whereas later steps include processes such as deposition of matrix, mineralization, and the formation of attachment bone (Huysseune et al., 1998). Therefore, replacement teeth that have just initiated during the start of the treatment slow down their development, likely because of reduced nutrient and oxygen supply. Replacement teeth with blood vessels in their pulp were likely already vascularized at the start of the treatment. Indeed, their developmental stage with respect to their predecessor suggests they were already large, and therefore vascularized, at the onset of treatment.

Interestingly, the mean distance between the closest point of the sinusoidal cavity and the

developing tooth germ is larger in delayed than in non-delayed replacement teeth. This relationship between an enlarged distance from the blood vessel and a delay in the development of the replacement tooth suggests the need for a proper blood supply to support ongoing tooth development (but not initiation) and endorses the hypothesis that we have previously raised on such a link (Crucke and Huysseune, 2013). We speculate that during odontogenesis, tooth germs express angiogenic factors attracting blood vessels towards the developing tooth. The pulpal expression of angiogenic factors, such as VEGF, has already been shown in human teeth (Mastrangelo et al., 2005, Miwa et al., 2008) and newts (Miwa et al., 2010), but which factors in zebrafish teeth attract (or repel) ingrowing blood vessels has not been addressed yet.

Our findings strongly suggest that the vasculature is likely not providing an inductive signal, i.e. not a trigger, for tooth replacement to start, but is rather involved in processes of maintenance and/or homeostasis. Studies in mice and rabbits that have addressed the role of vascularization in tooth eruption and resorption, have demonstrated a connection between the number of osteoclasts and the degree in which the dental and surrounding tissues are vascularized (Kohno et al., 2003, Motokawa et al., 2013). This hints towards a role in tooth resorption rather than tooth initiation. Zebrafish teeth are shed after resorption of the attachment bone by osteoclasts (Witten and Huysseune, 2009), whose precursors are likely supplied via the blood vessels. That we have not seen abnormal retention of functional teeth suggests that preventing new blood vessels to form is not sufficient to block supply of osteoclasts.

The developmental delay that we observe in the replacement tooth is a strong indication that the vasculature is responsible for providing the tooth germ with the necessary nutrients, oxygen and perhaps growth factors in order to develop in a timely fashion. The fact that first-generation teeth appear to develop unaffected in the absence of blood vessels may be linked to their very small size. Later generation teeth are much larger in size and would thus require the ingrowth of capillaries in order to still be able to sustain a sufficient supply of nutrients to keep growing and developing. Superficially, this resembles tumours not being able to increase in size without the ingrowth of surrounding blood vessels. Avascular tumours, for example, are severely restricted in their growth since they lack the proper blood supply. They must make an ‘angiogenic switch’ which tips the balance of proangiogenic and antiangiogenic factors in their favour (Folkman, 2001, Bergers and Benjamin, 2003). We speculate that the developing teeth in zebrafish must also make a similar ‘angiogenic switch’ securing the ingrowth of capillaries from the sinusoidal cavity allowing the teeth to fully grow and

develop.

Apart from an essential nutritive function and a possible involvement in tooth eruption and resorption, the vasculature may also have other functions. For example, it has been stated that perivascular cells, such as pericytes, are a possible source of mesenchymal stem cells (Crisan et al., 2008). Therefore, in the light of a continuously renewing dentition, the presence of a pool of stem cells at the base of the teeth seems an excellent feature in order to sustain and maintain the development of new teeth at frequent intervals. Previous research in mice and humans, has already shown the ability of pericytes to act as mesenchymal stem cells *in vitro,* especially with regard to their chondrogenic and osteogenic potential (Crisan et al., 2008, Zhao et al., 2014). Perivascular cells have been described in zebrafish (Santoro et al., 2009), and the possibility that the vasculature in the dentigerous region of zebrafish acts as a reservoir for mesenchymal stem cells cannot be excluded. The present study suggests that preventing new blood vessels to form was insufficient to block the pool of perivascular cells present. Furthermore, it has been shown that SU5416 has no effect on pericyte population (Bergers et al., 2003). Hence, pericytes could still potentially differentiate and initiate new tooth germs. This is in line with our findings since we have not stopped new tooth germs from developing but rather slowed down development.

Over recent years, the crosstalk between vascular and neural networks, termed the neurovascular link, has received more and more attention (Carmeliet, 2003b, Zacchigna et al., 2008a, Ulrich et al., 2011). This connection could also be of importance during the process of continuous tooth replacement. Nerves have already been shown to influence tooth replacement in *Tilapia mariae,* a polyphyodont teleost (Tuisku and Hildebrand, 1994). In addition, it has been shown in the skin of mice, that peripheral nerves provide a template for developing arteries to guide their growth and development towards their target (Mukouyama et al., 2002). Hence, the influence of the nervous system on both the vascular network and tooth development as a process itself cannot be neglected and needs to be elucidated.

3.6 CONCLUDING REMARKS

In conclusion, blood vessels do not appear to be required for the initiation of tooth development and replacement in the zebrafish. However, our data strongly suggest that the extensive network of blood vessels that surrounds later developing tooth generations, plays a role in supporting the appropriate environment for development and growth of the replacement teeth. Finally, it is clear that the link between the vasculature and innervation needs to be addressed if we wish to fully understand homeostatic processes governing the repeated formation of teeth in this model.

3.7. ACKNOWLEDGEMENTS

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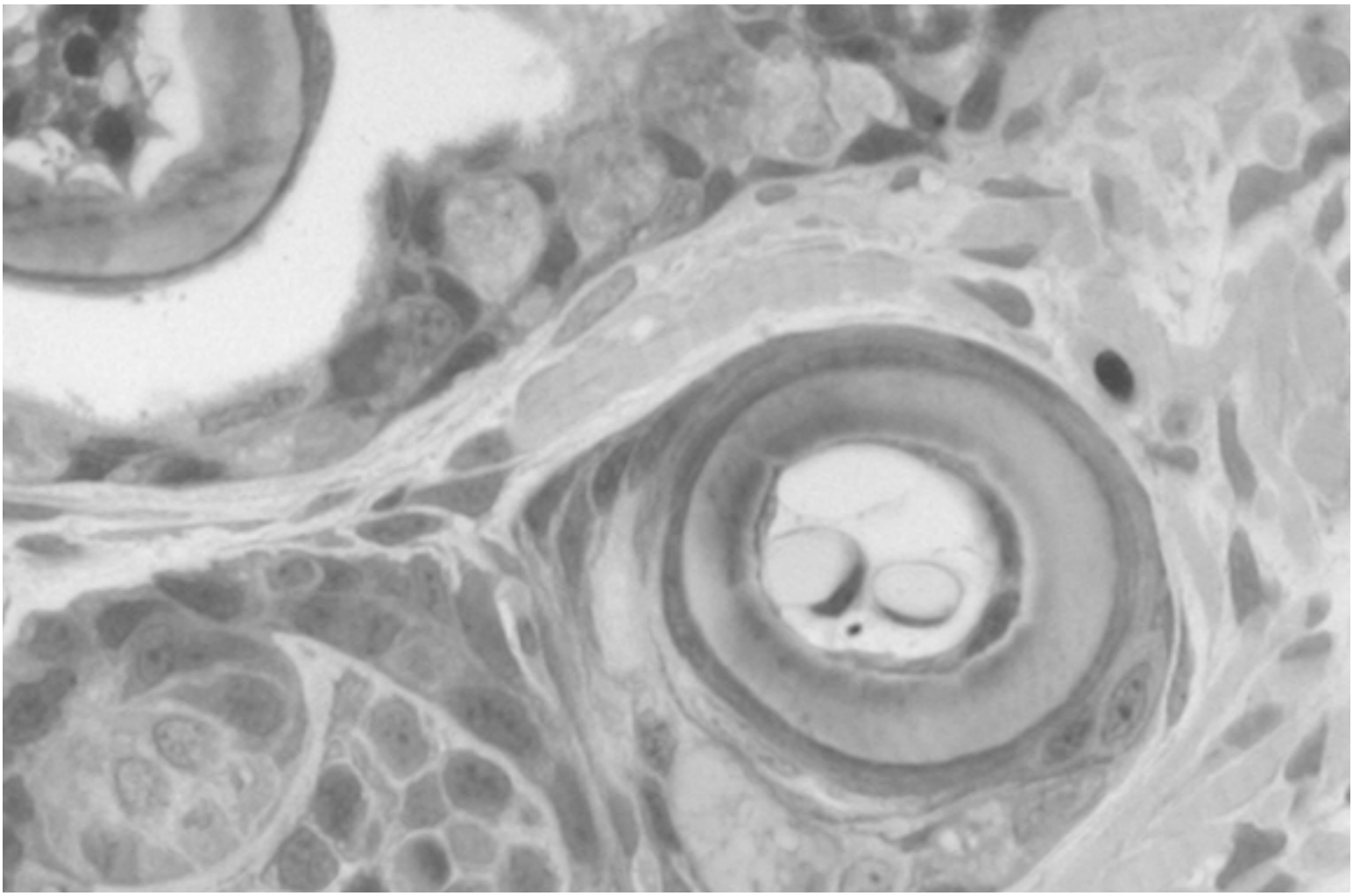
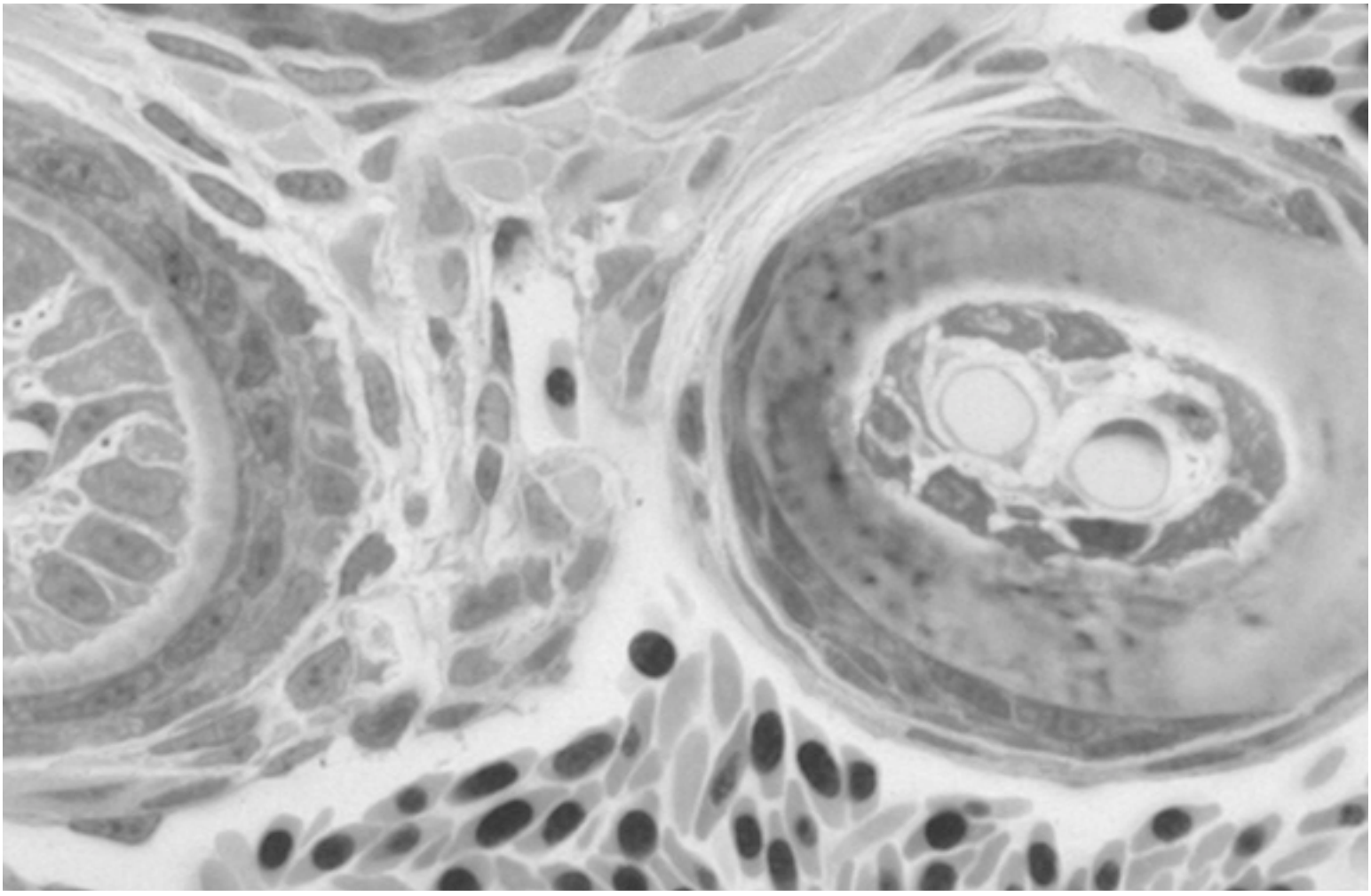


GENERAL DISCUSSION



4. GENERAL DISCUSSION

In this study we have attempted to find evidence for a possible neurovascular influence on the process of continuous tooth replacement in zebrafish. However, prior to studying a possible connection between blood vessels and/or nerves, and developing teeth, we first had to fill in the gaps with regard to the basic morphological information concerning vascularization and innervation of the zebrafish pharyngeal jaws (Figure 21). Our morphological approach has revealed the presence of a large sinusoidal cavity surrounding the dentition in zebrafish. The identification of this structure, however, has raised some important questions that will be discussed below. Furthermore, we have taken the first steps in elucidating the role of neurovascular elements on tooth development and replacement by studying the role of VEGF, an important angioneurin. Finally, we will discuss the possibilities for future studies in this field of research.

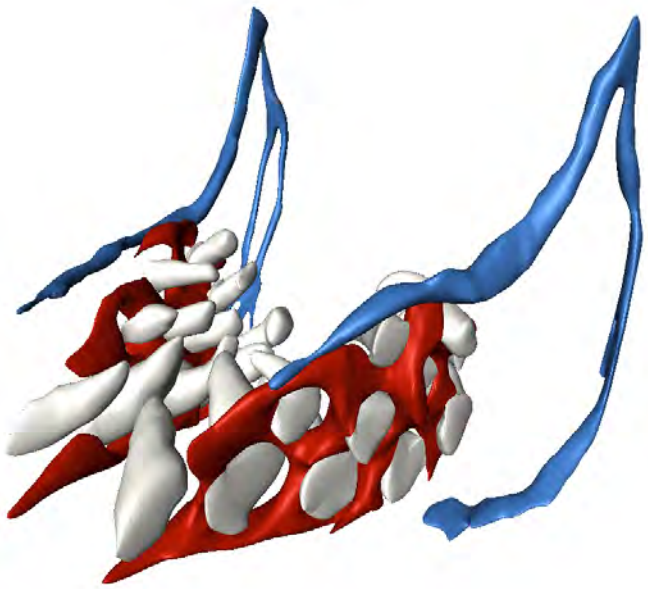


Figure 21: Vascularization and innervation of zebrafish teeth.
Visual representation of the zebrafish dentition (white) with the surrounding sinusoidal cavity (red) and the nerves (blue) responsible for innervating the pharyngeal jaws.

4.1 THE SINUSOIDAL CAVITY

We have identified a large vascular structure in the dentition of zebrafish, which we have termed the sinusoidal cavity. We have chosen this name since it concerns a large blood-filled sinus that anastomoses around the bases of both the functional and replacement teeth. The term 'sinusoidal', however, might be considered misleading as it has different meanings. Sinusoidal or discontinuous usually refers to the wall of capillary blood vessels and indicates the presence of fenestrations that allow the passage of even red and white blood cells (Satchell, 1991, Olson, 2000a, Olson, 2000b). For example, sinusoids are small blood-filled spaces or channels found in the tissue of organs such as liver, lymphoid tissue, endocrine organs, and hematopoietic organs such as the bone marrow and the spleen (Degroodt et al., 1957, Fu and Tcheng, 1966, Schmedtje and Batts, 1973, Satchell, 1991). Conversely, sinusoids also refer to a blood-filled space of irregular shape connecting arterial and venous capillaries (Satchell, 1991), and the term has been applied here accordingly. Given, however, the dual meaning of the term 'sinusoid', and thus, by extension, of the adjective 'sinusoidal', it may be useful to employ a different name, in future studies concerning this subject, such as 'dental blood sinus (DBS)'. This is in agreement with the term 'maxillary barbell blood sinus', recently used for a large blood filled chamber at the base of the barbell in zebrafish (Binelli et al., 2014). Indeed, in anatomy, a sinus usually refers to a hollow cavity either blood or air-filled. However, for the remainder of this discussion we will continue using the term 'sinusoidal cavity' as we have originally proposed in our publication describing the vascular anatomy of the dentigerous area in zebrafish (Crucke and Huysseune, 2013).

4.1.1 MORPHOLOGIES RESEMBLING THE SINUSOIDAL CAVITY

We wanted to know whether the presence of a sinusoidal cavity is unique to zebrafish, or whether similar morphologies, either tooth-associated or non-tooth-associated, can be found in other animals as well.

Tooth-associated structures

Our study in zebrafish revealed the presence of an unusual vascular complex in the dentigerous area. In chapter 1 of the results section, we proposed that this structure might be related to the phenomenon of continuous tooth replacement. However, to establish a possible connection to polyphyodonty, we needed to determine the distribution of this trait within the gnathostomes, and thus to investigate more species. Yet, in non-mammalians the vascular

supply to the developing teeth is poorly known and little information is available. Therefore, we will briefly introduce some preliminary data regarding the vascular supply of the dentition in two other taxa with continuous tooth replacement, highly relevant from a phylogenetic perspective: a chondrichthyan (*Scyliorhinus canicula*) and an osteichthyan (*Polypterus senegalus)*.

The lesser-spotted dogfish (*Scyliorhinus canicula*) is the most abundant species of catshark in European inshore waters and is found in the eastern Atlantic from Norway to West Africa, including the Mediterranean at depths of up to 400m (Jawad, 2013). The catshark is positioned within the Chondrichthyes, sister group to the Osteichthyes, with whom they share both derived (apomorphic) and ancestral (plesiomorphic) traits. The African bichir (*Polypterus senegalus*) is a freshwater fish native to large rivers, estuaries, and lakes in tropical Africa and a member of the earliest diverged actinopterygian group with living representatives (Bartsch et al., 1997, Near et al., 2012). Its dentition has been well characterized in terms of distribution and tooth shape (Clemen et al., 1998, Wacker et al., 2001). For both *S. canicula* and *P. senegalus* information regarding the vascular supply to the dentigerous region is for the largest part lacking. Histological study and (partial) reconstruction of the dentition along with the surrounding vasculature in both species, revealed the presence of a large thin-walled vascular structure, much as we described in zebrafish (Figure 22). In both *S. canicula* and *P. senegalus* branches can be seen issuing from this structure, and securing the intrapulpal supply of blood. In addition, the blood vessels seem to surround the developing teeth, either partially (*P. senegalus*) or completely (*S. canicula*). Given these similarities to what we have described in zebrafish, we can rightfully state that in both species blood is secured to the teeth through the presence of a sinusoidal cavity in the dentigerous region. Thus, a similar vascular structure occurs not only in two species of ray-finned bony fish, but also in a cartilaginous fish, indicating that the sinusoidal cavity is not unique to zebrafish.

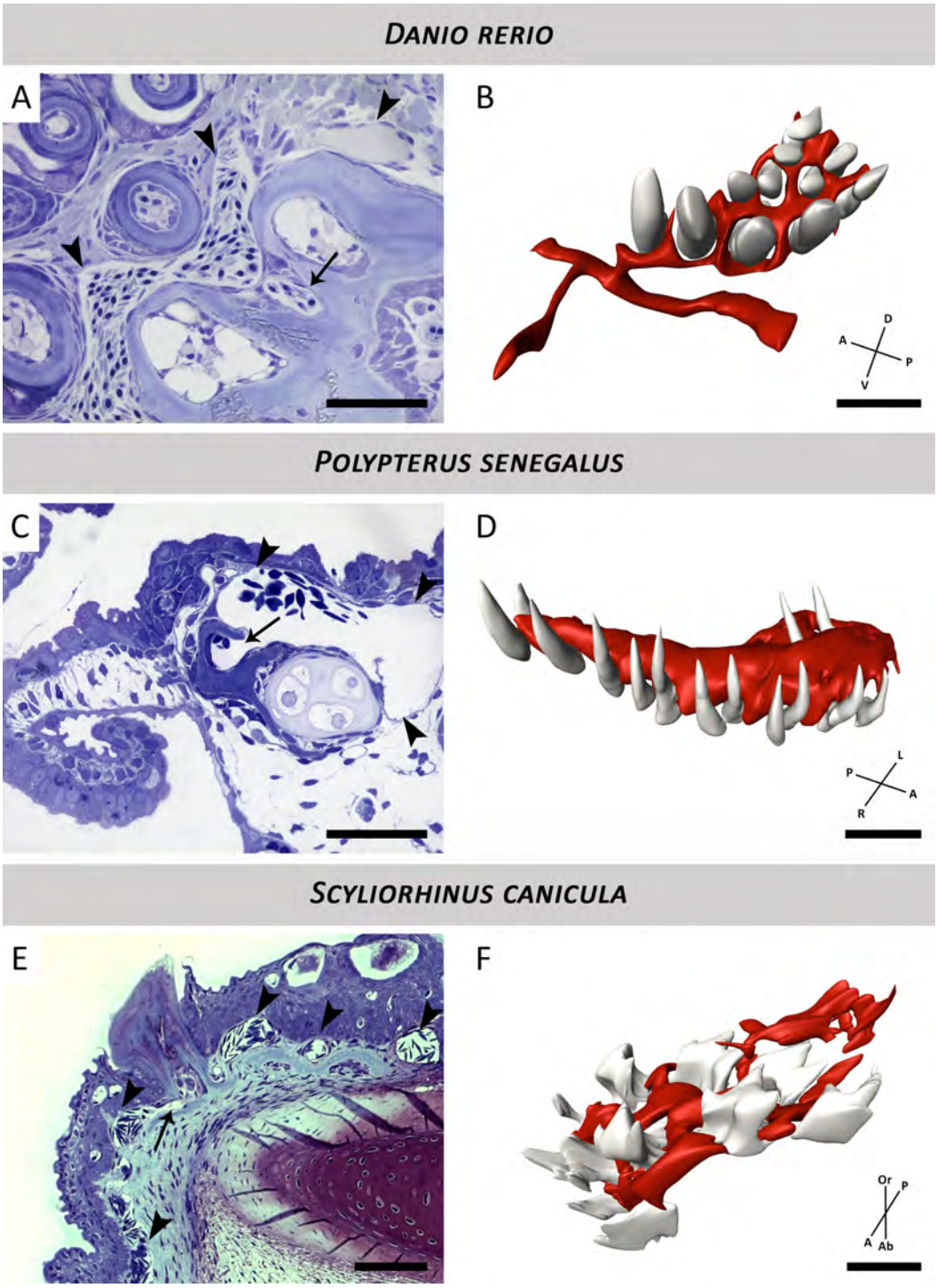


Figure 22: Vascularization pattern in the dentigerous region of 3 different species.
(B, D, F) 3D reconstructions visualizing the vascular pattern (red) and the surrounding teeth (white) in three different species with continuous tooth replacement, *Danio rerio* (SL 9.5 mm), *Polypterus senegalus* (TL 10 mm), and *Scyliorhinus canicula* (TL 11.7 cm). For each reconstruction, one of the sagittal (A) or transverse (C, E) toluidin blue stained histological sections is included on wich the 3D model is based (A, C, E). Note an elaborate thin-walled vascular cavity (arrowheads) in all three species giving rise to branches (arrows) securing the intrapulpal blood supply. Orientation: A: anterior, P: posterior, D: dorsal, V: ventral, L: left, R: right, Or: oral, Ab: aboral. Scale bars: 50 µm (A-D), 100 µm (E, F).

Despite this seemingly similar sinusoidal cavity in all three species, some differences must be pointed out. Zebrafish possess only pharyngeal teeth that are implanted on the fifth ceratobranchials, whereas the teeth studied in both *P. senegalus* and *S. canicula* are oral teeth implanted on the dentary bone and Meckel's cartilage respectively. Hence, differences can be expected in terms of the origin of the blood supplying the dentigerous region. In zebrafish, we have determined the blood supply to be coming from the hypobranchial artery, more specifically from the coronary artery branching off from the hypobranchial artery. This may not be surprising given the position of zebrafish teeth just dorsal of the heart. Although studies concerning the vascular supply to the teeth in both *P. senegalus* and *S. canicula* are scarce, based on descriptions of the vascular anatomy in these species, we can assume the blood supply to the oral teeth to be secured by either the hypobranchial artery or the mandibular artery (Allis, 1922, de Beer, 1924). The hypobranchial artery represents an ideal candidate for supplying blood to developing teeth in general for chondrichthyans and osteichthyans, since it arises from the first aortic arch and runs posteriorly towards the heart. Thus, the hypobranchial artery covers the entire region where teeth are potentially present. In this way, the observed differences in position of teeth between *D. rerio*, *P. senegalus*, and *S. canicula*, do not necessarily have to be reflected in differences in the vascularization pattern. However, given the lack of data regarding the vascular supply of teeth in aquatic non-mammalians, this needs to be confirmed.

Non-tooth-associated structures

Other structures can be found that superficially resemble the sinusoidal cavity as we have described for *D. rerio*, *P. senegalus*, and *S. canicula*, but that are not associated with dental structures. A recent study has demonstrated the presence of a large blood sinus at the basis of the maxillary barbell of zebrafish (Binelli et al., 2014). However, different from the sinusoidal cavity in the dentigerous area, this endothelial chamber at the base of the barbell consists of three large parts (distal bulb, central chamber, and accessory chamber), each with its own neurovascular specializations. The authors propose that this new organ in zebrafish might be involved in sensing its environment along with the control of barbell movement (Binelli et al., 2014). Analogously, in mammals, vibrissae are associated with a prominent blood sinus that is heavily innervated by sensory neurons, acting together as a sensory system (Rice et al., 1986, Ebara et al., 2002). In rats, this vibrissal follicle-sinus complex consists of a superior portion, known as the ring sinus, and an inferior portion, known as the cavernous sinus (Kim et al., 2011). These studies suggest that apart from a possible connection of the sinusoidal

cavity in zebrafish to tooth development and replacement, a role as a mechanosensory system cannot be excluded.

4.1.2 Additional roles of the sinusoidal cavity

Previously we have already discussed some possible functions of the sinusoidal cavity, e.g. nutritional, and/or a potential pool of mesenchymal stem cells (See results section, chapter 1). However, taking into account the preliminary data that we have shortly introduced from *S. canicula* and *P. senegalus* in the previous section, we can speculate further on a possible correlation between the sinusoidal cavity and polyphyodonty. Finally, the vasculature might also function in bone remodelling processes during tooth development.

Connection to polyphyodonty

Given the presence of a sinusoidal cavity in three different extant species, namely *D. rerio*, *P. senegalus*, and *S. canicula*, and given their phylogenetic relationships (Figure 23), the origin of the sinusoidal cavity can be traced back to a structure present in the common ancestor of the Osteichthyes and Chondrichthyes. Alternatively, the sinusoidal cavity might have arisen several times independently and represent a case of convergent evolution.

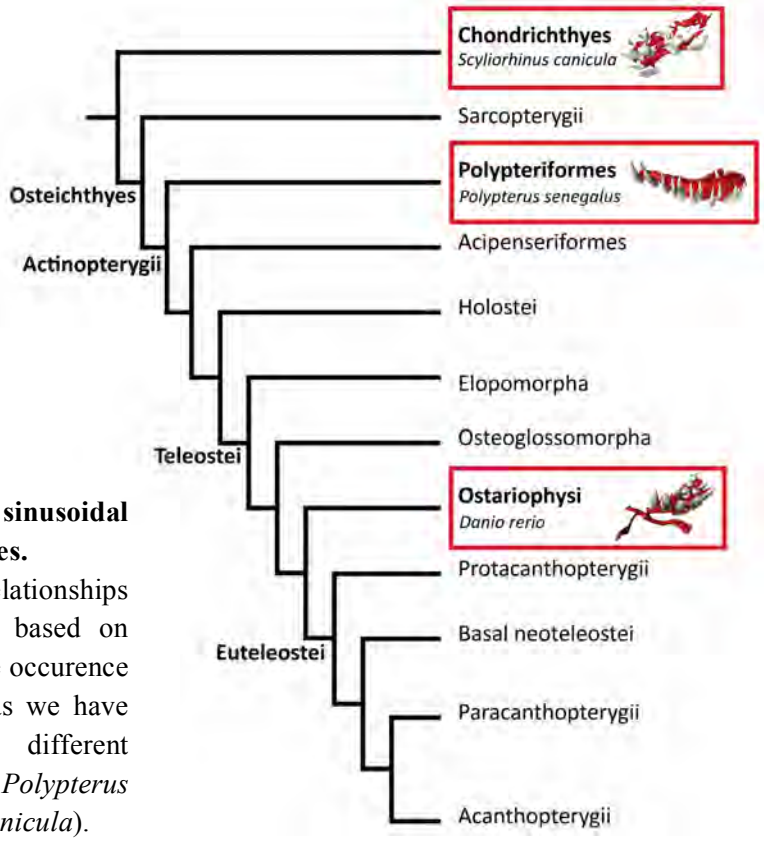


Figure 23: Occurence of sinusoidal cavity in living vertebrates.
Simplified phylogenetic relationships within the gnathostomes based on Near et al. (2012), and the occurence of the sinusoidal cavity as we have demonstrated in three different species (*Danio rerio*, *Polypterus senegalus*, *Scyliorhinus canicula*).

Given that all species that possess a sinusoidal cavity around the teeth display continuous tooth turnover, we speculate that the sinusoidal cavity might represent an evolutionary adaptation of the vascular system allowing the continuous replacement of teeth. Moreover, we hypothesize the reduction/loss of the sinusoidal cavity to have played a role in the transition from polyphyodonty to mono/diphyodonty. Studying the blood supply to the developing teeth in representatives with continuous tooth replacement from the Sarcopterygii could provide evidence in favour of this hypothesis. While data regarding the vascular supply during odontogenesis in amphibians or reptiles is scarce, published histology data on odontogenesis in these species allows us to make some preliminary assumptions. In amphibians, one of the earliest diverged sarcopterygian groups, published data regarding tooth development in living representatives shows the presence of large blood vessels in the mesenchyme surrounding the developing teeth, although to a lesser extent to what we have described in zebrafish, (Davit-Beal et al., 2007, Davit-Beal and Sire, 2007). Histology data from reptiles such as snakes (Buchtova et al., 2008, Handrigan and Richman, 2010b), lizards (Handrigan and Richman, 2010a), and crocodiles (Wu et al., 2013) on the other hand, do not appear to show a similar extensive vascularization of the dentition. Based on these observations, the amount and extent of vascularization of teeth seems to have decreased in the amphibian lineage, and even more within reptiles. This seems to contradict a connection between the sinusoidal cavity and continuous tooth replacement. So why do some polyphyodont species possess this vascular structure while others do not? Several hypotheses might be proposed to explain these observed differences. A first hypothesis to be tested is based on hydrodynamics. The tooth-bearing region of the jaws is a highly dynamic region: teeth have to be constantly broken down and rebuilt, and the subjacent bone has to be continuously remodelled accordingly. Hence, there is a constant need of (blood-borne) osteoclasts, of means to transport resorbed components, of building blocks to construct new teeth, and of oxygen to fuel these processes. Given the thin-walled nature of the wall of the sinusoidal cavity, and the need of sufficient oxygen and nutrients in such a highly dynamic environment, we can safely assume that the sinusoidal cavity functions in exchange. In order to allow sufficient exchange with the tissues, the blood must flow sufficiently slow (Pries et al., 1996). In general, this is achieved by the branching of a large vessel (arteriole) into multiple smaller vessels (capillaries) since the sum of the cross sections of the capillaries is larger than that of the arteriole, and according to laws of hydrodynamics, this slows down the blood stream. Alternatively, enlarging a vessel into a large chamber must likewise reduce the speed of the blood flow and facilitate exchange. So why choose the latter possibility over the presence of multiple capillaries? We can only guess.

However, given the highly dynamic nature of the tissues involved we may assume the first option, the presence of a capillary network, would entail also a constant remodelling of such a network. The vicinity of a constantly present sinusoidal cavity from which capillaries branch off 'on demand' appears to be a more parsimonious solution than the constant modification of the whole capillary network. However, this would suggest that the sinusoidal cavity is closely associated to continuous tooth replacement. Since there does not seem to be a sinusoidal cavity in reptiles, where there must be an important tooth turnover as well, this can only be a partial explanation, and we need to raise also a second hypothesis.

The second hypothesis to explain the presence of a sinusoidal cavity is in the nature of the circulatory system in the different vertebrates concerned. Both chondrichthyans and actinopterygyians posses a single circuit for blood flow, where blood returning from the body is collected in the atrium and pumped to the gills by the ventricle where it is re-oxygenated. This unidirectional flow of blood produces a gradient of oxygenated to deoxygenated blood around the organism's systemic circuit. The result is a limit in the amount of oxygen that can reach some of the organs and tissues of the body, reducing the overall metabolic capacity of fish (Satchell, 1991). Hence, if the blood that reaches the teeth contains lower levels of oxygen after passage over the heart, via the coronary artery, one may speculate that a large sinusoidal cavity favouring ample exchange compensates for the poor oxygen content of the blood. In reptiles, which have at least a partial double circulatory circuit, one through the lungs and back to the heart (pulmonary circulation) and the other throughout the rest of the body and its organs (systemic circulation) (Withers, 1992), the teeth are supplied by oxygen-rich blood and constraints on means for gas exchange may be more relaxed. Caudate amphibians possess teeth both before and after metamorphosis (Wistuba et al., 2002). Before metamorphosis, there is a single circuit using external gills, after metamorphosis the circuit is partially double through the development of lungs. Examining the presence of a sinusoidal cavity before (when oxygenation occurs in the gills) and after metamorphosis (when oxygenation occurs in the lungs) would thus provide a very nice test for this second hypothesis.

At present, the prevailing hypothesis to account for the loss of multiple tooth generations in the reptile-to-mammal transition is based on the loss/regression of the dental lamina in diphydont species after the development of the second-generation teeth (Buchtova et al., 2012, Whitlock and Richman, 2013). Ample evidence supports this hypothesis. In humans and minipigs, the dental lamina undergoes degradation after the initiation of the second-

generation teeth, preventing the development of further tooth generations (Moskow and Bloom, 1983, Stembirek et al., 2010). Incomplete dental lamina degradation in humans has been linked to the formation of epithelial pearls, which can lead to the formation of oral cysts and tumours (Moskow and Bloom, 1983, Eversole, 1999). In addition, the presence of a third generation dental lamina has been reported once in humans originating from the dental organ of the mandibular permanent lateral incisor (Ooë, 1981), a position where supernumerary teeth often form (Wang and Fan, 2011). Mechanisms involved in lamina degradation have been proposed to include basement membrane breakdown, epithelial to mesenchymal transition (EMT), and apoptosis (Buchtova et al., 2012). Nonetheless, the presence of a sinusoidal cavity in the dentigerous region of *D. rerio*, *S. canicula*, and *P. senegalus* hints for the first time towards a possible connection between the vasculature and polyphyodonty. The distribution of such a vascular structure in sarcopterygians should therefore be taken into account when discussing the evolutionary transition from polyphyodonty to diphyodonty.

Connection to bone remodelling during tooth development

The role of the vasculature during processes such as tooth eruption and resorption has already been studied in mammalian teeth. Studies performed on mice and rabbits have demonstrated a connection between osteoclastic activity and the degree in which the surrounding dental tissues are vascularized (Miller, 1957), indicating a role for the vasculature in tooth resorption. In rats, dogs, and rhesus monkeys, remodelling of the periodontal ligament through application of experimental force is achieved through activation of the vascular system. Increased vascular invasion was observed on the sites of tension resulting in higher rates of bone resorption (Khouw and Goldhaber, 1970, Rygh et al., 1986). Penetration of blood vessels is likely achieved through the expression of VEGF in osteoblasts on the tension side as was demonstrated in mice (Kohno et al., 2003). Hence, the vasculature seems to play an important role in delivering osteoclasts to bone remodelling sites. In zebrafish, mononucleated osteoclasts (juveniles) precede multinucleated osteoclasts (adults) and are responsible for resorption of the attachment bone prior to shedding of the tooth (Van der heyden et al., 2000, Witten et al., 2001). A role for the vasculature in tooth resorption in zebrafish thus seems likely.

4.1.3 INTRAPULPAL BLOOD FLOW

One point which we have not yet previously raised in our discussion of the sinusoidal cavity is how blood flows within the teeth of zebrafish. We have described the presence of capillaries branching off from the sinusoidal cavity, hence securing the intrapulpal supply of blood (Crucke and Huysseune, 2013). However, as we observed the presence of several smaller capillaries merging with the branch issuing from the sinusoidal cavity securing the intrapulpal supply, we were not able to unequivocally determine the course of the pulpal blood vessels (see Figure 10). The pulpal vessels might either reconnect to the sinusoidal cavity or drain into veins. If the blood reconnects to the sinusoidal cavity, this raises the question as to how blood flow is generated within the tooth.

In humans, blood is supplied to the teeth by branches of the maxillary artery. More specifically, the blood supply of the upper jaw teeth has its origin in the posterior superior alveolar arteries and the infraorbital artery, whereas the blood supply to the lower jaw teeth is secured by the inferior alveolar branch of the internal maxillary arteries (Meyer et al., 1964). Arterioles branching from these arteries enter the pulp through the apical foramina. Arterioles will then eventually turn to capillary beds serving exchange, which will eventually connect to venules that will exit the tooth (Scheinin, 1963). The walls of arterioles and venules are coated with smooth muscle cells, innervated by both myelinated and unmyelinated sympathetic fibres. When stimulated, the muscle fibres contract, thus decreasing the diameter of the vessel and reducing blood flow (Edwall and Kindlova, 1971). In addition, pulpal blood flow is also greatly influenced by the low-compliance environment in the pulp, due to the encasement within the dentin. Hence, vasodilation of the arterioles in the pulp might cause compression of the venules thus reducing pulpal blood flow (Heyeraas, 1989).

In zebrafish similar mechanisms are possibly responsible for regulating blood flow. Indeed, the presence of cells potentially corresponding to pericytes, have been observed in the dentition of zebrafish (see further), and we have demonstrated the presence of nerve fibres in the pulp of functional teeth (Crucke et al., 2015). In addition, similar to humans, the microvasculature of the teeth is encased in a rigid dentin structure creating a low compliance environment, thus indirectly influencing blood flow. These mechanisms hold if the outflow of the teeth connects to veins (e.g. anterior cardinal vein). However, if the capillaries inside the tooth reconnect to the sinus, blood inside the tooth is probably stagnant as both in and outflow are originating from the same vessel. Further research is required to clearly determine where and how blood flows within the teeth of zebrafish.

4.2 EVIDENCE FOR A NEUROVASCULAR LINK?

In general, the presence of a neurovascular link can be inferred based on anatomical evidence, as well as shared cellular and molecular pathways (Carmeliet and Tessier-Lavigne, 2005, Zacchigna et al., 2008a, Segura et al., 2009). Below, we will discuss possible anatomical evidence for the presence of a neurovascular link in zebrafish, in addition to focussing on the functional connection between a neurovascular link and tooth development.

Anatomical evidence

The vascular supply of the teeth in zebrafish originates from the hypobranchial artery located on the ventral side of the fifth ceratobranchials. Upon reaching the dentition, functional teeth become surrounded by an elaborate sinusoidal cavity, giving off branches towards the developing teeth and securing the intrapulpal supply of oxygen and nutrients. An internal branch of a posttrematic ramus of the vagal nerve on the other hand secures the nervous supply. This ramus connects to a large vagal sensory ganglion on the dorsal side of the dentition. Initially we had also raised the hypothesis of a possible involvement of the enteric nervous system in the innervation of zebrafish teeth given their location deep within the pharynx. This seems unlikely since the intestinal ramus of the vagal ganglion runs caudally away from the dentigerous region.

Superficially, both vascular and neural networks do not appear to line up for wiring of the dentition in zebrafish, at least not at a gross anatomical level (Figure 1). If blood vessels were to guide developing neurons or vice versa we would expect the branching pattern of nerves to mimic that of vessels, similar to what has been demonstrated in the skin of mice (Mukouyama et al., 2002), or in the forelimb of quail embryos (Bates et al., 2002). However, alignment of blood vessels and nerves might still occur at each individual tooth position between smaller branches of both vessels and nerves running towards the pulp. We have shown that penetration of both capillaries and nerves into the dental pulp is achieved only during late stages of odontogenesis, hinting towards a coordinated development between the systems. Hence, if colocalization of capillaries and smaller nerve branches occurs, it might be achieved through the expression of growth factors in developing tooth germs. Different scenarios can be proposed how such a colocalization could be achieved. The developing tooth germ could express growth factors, such as angioneurins, attracting both vessels and nerves, resulting in their ingrowth and similar patterning. An obvious candidate in order to achieve this is VEGF, since it has been shown to serve numerous functions in the nervous system (Rosenstein et al.,

2010, Mackenzie and Ruhrberg, 2012) as well as being a key regulator in the guiding of endothelial tip cells during angiogenesis (Carmeliet et al., 1996b). The pulpal expression of VEGF has already been demonstrated in human teeth (Mastrangelo et al., 2005, Miwa et al., 2008), and newts (Miwa et al., 2010). The involvement of VEGF in attracting vessels and/or nerves towards developing teeth can thus be expected, but has not yet been confirmed in zebrafish. On the other hand, developing tooth germs might also express neurotrophic factors attracting nerves, which in turn express angiogenic factors that initiate the development of new branches from the sinusoidal cavity towards the teeth. This would be similar to a certain extent to what has been observed in the skin of mice (Mukouyama et al., 2002), where nerves provide a template for arteries by guiding their development through the expression of VEGF. The expression of neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), in developing tooth germs has been demonstrated in the dental papilla/pulp of rats (Byers, 1990, Luukko et al., 1997, Nosrat et al., 1998) and in developing human teeth (Christensen et al., 1993, Nosrat et al., 2002). However, in zebrafish, information regarding the involvement of neurotrophic factors in odontogenesis is completely lacking. Determining the order in which both the vascular and neural system penetrate the tooth pulp would aid in elucidating whether nerves guide vessels or vice versa during zebrafish tooth development. From an evolutionary perspective, one could expect nerves to provide a template for developing arteries since it is generally believed that the nervous system arose prior to the vascular system (Miller, 2009). In addition, distant homologs of mammalian VEGF and VEGFRs were already detected in invertebrates lacking a vascular system (Cho et al., 2002), providing further support for a guiding role of the nervous system for the vasculature.

Functional link to tooth development

Apart from collecting anatomical evidence to test the possible involvement of a neurovascular link during tooth development and replacement, we have also undertaken the first steps in demonstrating a possible functional connection between vascular elements and odontogenesis. We have looked at the effect of the vasculature on the initiation of both first-generation and replacement teeth through interfering with the VEGF signalling pathway, hence arresting the development of new blood vessels. Results show that, while new teeth are still being initiated, replacement teeth developing during the time of treatment clearly show a developmental delay. In our study, however, we have only prevented *de novo* development of blood vessels to newly forming tooth germs. We did not remove the existing vasculature. Since replacement

teeth that were delayed showed a larger distance to the nearest vascular elements (the sinusoidal cavity) than replacement teeth that were not delayed, we propose that the observed delay is most likely due to a decreased supply of nutrients and oxygen to the developing tooth germs. Nonetheless, new replacement teeth were still initiated. Interestingly, perivascular cells such as pericytes have recently been proposed as a source of mesenchymal stem cells (Crisan et al., 2012). In addition, not only perivascular cells, but also peripheral nerve-associated glia have been shown to act as a possible source of multipotent mesenchymal stem cells producing pulp cells and odontoblasts, at least in the continuously growing incisor of mice (Kaukua et al., 2014). In zebrafish, the presence of pericytes has not yet been demonstrated in the dentigerous region. However, preliminary observations of our semithin sections reveal the presence of cells that are potentially pericytes (Figure 24). Transmission electron microscopy is necessary to firmly establish the identity of these potential candidates as pericytes.

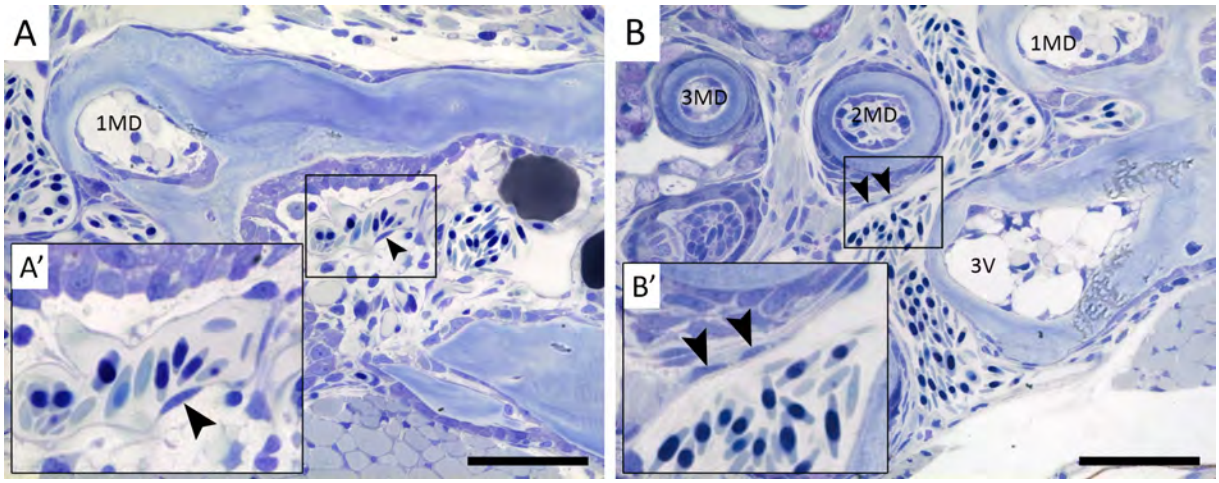


Figure 24: Potential pericytes in the dentition of zebrafish.
Sagittal toluidin blue stained histological sections of a WT zebrafish (SL = 9.5mm) showing the presence of cells that are potentially pericytes (arrowheads, A, B). (A', B') Enlargement of the areas indicated in A and B respectively. Scale bars: 50 µm

Recent studies on the mouse incisor model have identified the neurovascular bundle present in the incisor as a mesenchymal stem cell niche (Zhao et al., 2014). These authors demonstrate the secretion of sonic hedgehog (Shh) protein by sensory neurons, which in turn activate Gli1 expression of periarterial cells in the bundle that eventually contribute to all mesenchymal derivatives. Clearly, if tooth turnover depends on a source of mesenchymal cells, blocking angiogenesis did not compromise the latter. Moreover, epithelial stem cells have also been proposed as having a key role in the process of continuous tooth replacement

(Huysseune and Thesleff, 2004). Ongoing research indeed focuses on elucidating the potential role of epithelial stem cells during continuous tooth replacement and identifying possible stem cell niches in both actinopterygians and sarcopterygians (Harada and Ohshima, 2004, Buchtova et al., 2008, Handrigan et al., 2010, Vandenplas et al., 2014). If the presence of stem cells is both necessary and sufficient to trigger tooth replacement, neurovascular elements in the vicinity of the functional tooth may still be involved in providing mesenchymal stem cells, even when sprouting of new vessels is blocked. Tuisku and Hildebrand (1994) have highlighted the importance of nerves during odontogenesis by demonstrating an arrest in tooth replacement in the teleost *Tilapia mariae* through transection of the trigeminal nerve. Hence, removing the neural component might either have succeeded in removing the potential source of mesenchymal stem cells for tooth initiation (e.g. nerve-associated glia), or in removing the inductive signal produced by nerves to induce the differentiation of periarterial cells as was demonstrated in the mouse incisor (Zhao et al., 2014). The reciprocal interaction between vessels and nerves emphasizes the importance of the neurovascular link during odontogenesis and shows that further research into this subject is required.

4.3 FUTURE PERSPECTIVES

It is clear that our study has undertaken only the first essential steps in studying the role of the neurovascular link during tooth development and replacement in zebrafish. In order to fully understand the significance of the sinusoidal cavity, or the role of the neurovascular link in odontogenesis and continuous replacement, further research is required.

Sinusoidal cavity and polyphyodonty

The identification of the sinusoidal cavity in three species, and its possible significance from a physiological as well as an evolutionary perspective has opened new avenues for future research. On the one hand, future studies should focus on studying the vascularization of teeth from representatives of different lineages with continuous tooth replacement. On the other hand, studying the vascularization pattern of the teeth in e.g. caudate amphibians before and after metamorphosis might yield relevant information regarding the connection between the sinusoidal cavity and the type of circulatory system (single or double).

Studies in zebrafish mutants possessing supernumerary teeth (Stock et al., 2006, Seritrakul et al., 2012, Aigler et al., 2014) might also yield important information regarding the role of the vasculature in evolutionary changes in number, position, or cycling of teeth. In zebrafish, overexpression of the Ectodysplasin (Eda) signalling ligand restores teeth to the upper pharynx (Aigler et al., 2014), whereas exogenous treatment with RA causes a dramatic expansion of the pharyngeal teeth to more anteriorly positioned arches (Seritrakul et al., 2012). These cases provide nice examples to study the connection between vascularization and teeth in zebrafish. Provided these supernumerary teeth undergo replacement, and assuming that vascularization plays a role in the further development of replacement teeth, we expect the supernumerary teeth in both cases to be similarly vascularized, as is the case for the pharyngeal teeth on the fifth ceratobranchials.

Sinusoidal cavity and intrapulpal blood flow

We have not been able to firmly establish how blood flows within zebrafish teeth in our current study. Future studies could therefore focus on elucidating the intrapulpal blood flow. A method for the *in vivo* study of blood flow through injection of small (less then 1 μm) tracer particles has been optimized for zebrafish (Craig et al., 2012). This method has already been routinely applied for blood flow mapping with no adverse effects on zebrafish physiology or survival. The difficulty, however, in studying intrapulpal blood flow of zebrafish teeth would be the size and location of the teeth. Given their location deep within the pharyngeal cavity, visualization of fluorescent beads would prove difficult, even more so since we are dealing with mineralized tissues such as teeth. However, techniques such as two-photon excitation fluorescence and second harmonic generation microscopy show potential and might be considered for the *in vivo* study of the dentition in zebrafish (Bruneel et al., 2015). Hence, these visualization methods in combination with the injection of microfluorescent tracer particles might provide better insights in the intrapulpal blood flow of zebrafish teeth.

Neurovascular link during odontogenesis

One important line of research should focus on identifying growth factors that potentially tie tooth formation to neurovascular structures and/or processes. We have taken the first preliminary steps in this process by interfering with VEGF signalling, an important angioneurin. It is clear that the expression pattern of the VEGF gene, and the protein distribution, can help to identify the importance of VEGF in establishing a correct

neurovascular environment for the developing teeth. In addition, we should not only focus on VEGF, but also study other growth factors that have already been proposed as important angioneurins such as NGF, PDGF and EGF (Carmeliet and Tessier-Lavigne, 2005, Zacchigna et al., 2008a). Comparing expression data from these angioneurins, their protein distribution, and function through chemical inhibition or (conditional) deletion, should allow us, or others, to propose hypotheses with regard to their potential function during tooth development and replacement.

Another line of research should make use of the wide array of mutants and transgenic lines readily available for zebrafish. Studying and visualizing the neurovascular link might therefore be achieved by combining a vascular-specific transgenic line with immunohistochemical staining for nerves with a primary antibody against acetylated tubulin. Appropriate transgenic lines include Tg(fli1:EGFP) and Tg(kdrl:GFP) where the promotor of fli1 (an endothelial marker), and kdrl (VEGFR2), respectively, drive the expression of green fluorescent protein (GFP). This is similar to the approach used to study the neurovascular development of the hindbrain in embryonic zebrafish (Ulrich et al., 2011). However, even better would be to make a double transgenic line with red fluorescence for blood vessels (e.g. Tg(flk:mCherrry)) and green fluorescence for nerves (e.g. Tg(isl1:GFP)), to visualize both networks simultaneously. In addition, the use of zebrafish mutants could aid in determining whether vessels precede or follow nerves in penetrating the teeth. If nerves still develop correctly in vascular-specific mutants then the development of both systems can be considered to be independent. A possible candidate for such an approach is the vascular mutant *out of bounds* (*obd*), which carries a mutation in the obd gene that is crucial for vascular patterning. Thus, *obd* mutant embryos display premature and incorrect vessel sprouting (Childs et al., 2002).

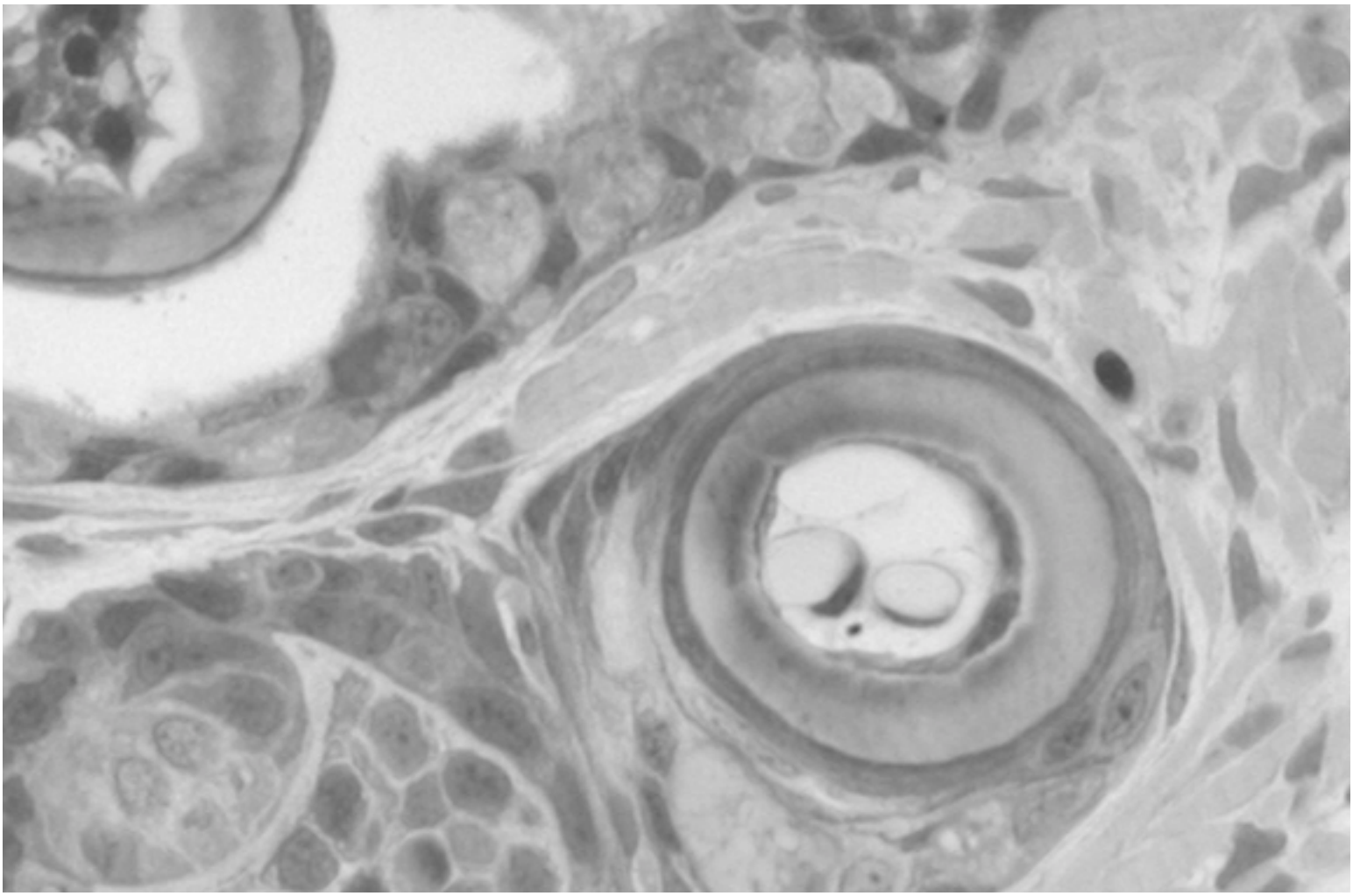
4.4 CONCLUDING REMARKS

While the research discussed in this thesis is fundamental, it is possible to reflect on outcomes that could potentially be of a more applied nature. For example, in the field of regenerative dentistry one hopes to achieve a so-called ‘tertiary dentition’. However, at present, it is not yet possible to regrow or regenerate whole teeth *in situ*. Nonetheless, the enormous progress of knowledge with regard to the genetic and molecular mechanisms that regulate (mammalian) tooth development (Jernvall and Thesleff, 2000, Thesleff, 2003, Tucker and Sharpe, 2004,

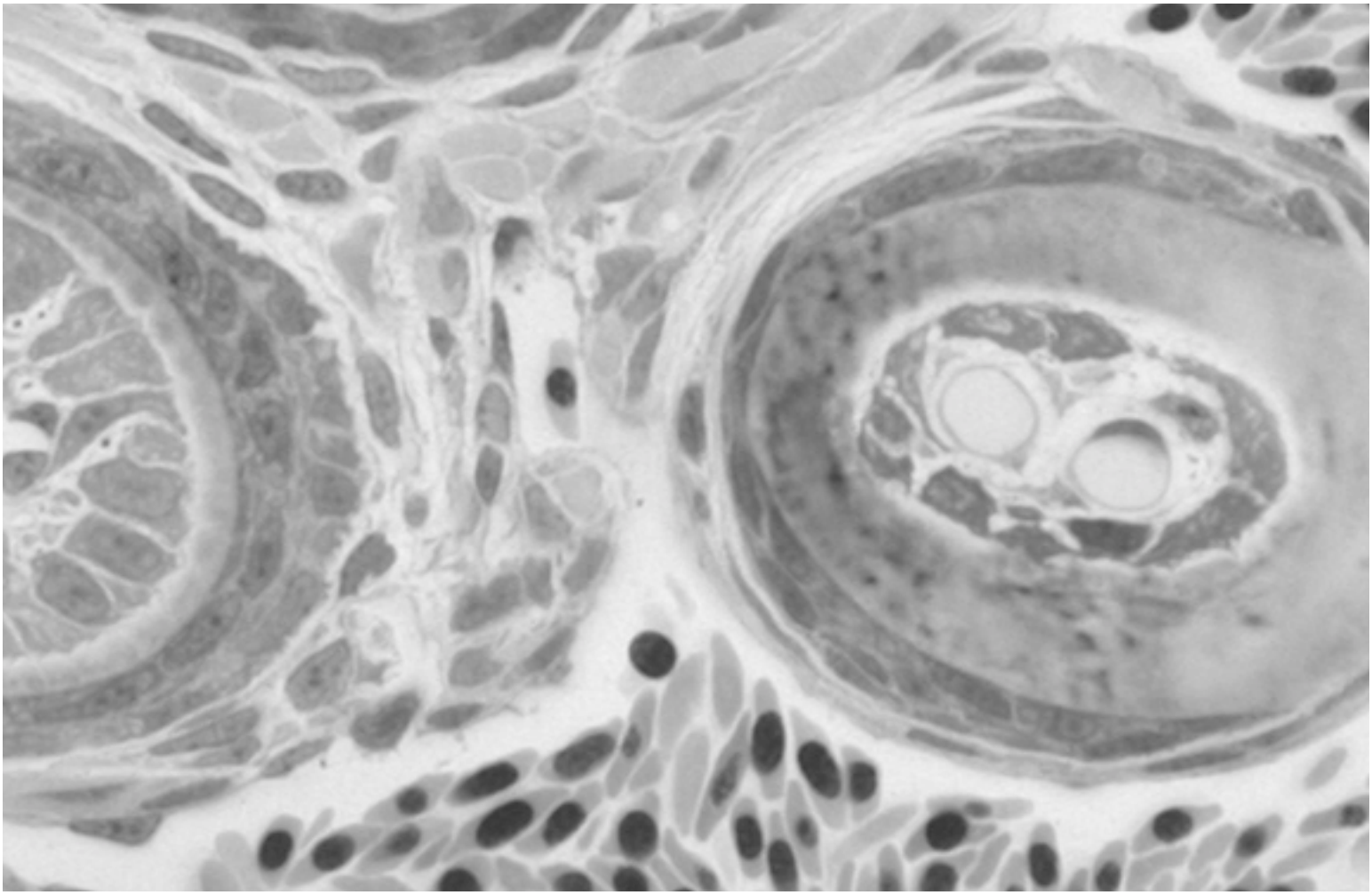
Klein et al., 2006, Prochazka et al., 2010) has advanced the field of regenerative dentistry up to the point where the engineering of teeth is getting within reach (Nait Lechguer et al., 2008, Galler and D'Souza, 2011, Kokten et al., 2014, Otsu et al., 2014). The process of tooth regeneration is complicated by the nature of the tooth itself, an intact tooth consisting of different tissues (mesenchymally derived pulp, dentin, and epithelially derived enamel) (Scheller *et al.*, 2009), as well as by the environment in which it has to grow and be maintained. In order to achieve full development, attachment and maintenance of teeth that can also fulfil their sensory function, proper vascularization and innervation must inevitably accompany their development. In the field of tissue engineering, the importance of an improved vascularization and innervation of the regenerated tissues has been addressed before, and still is a topic which needs to be further elucidated (Griffith and Naughton, 2002, Nomi et al., 2002, Nait Lechguer et al., 2008, Loai et al., 2010).

In this study we have undertaken the first steps in addressing, in future studies, the role of the neurovascular link during natural tooth replacement in zebrafish. In our descriptive approach we have identified both the neural and vascular supply to the fifth tooth bearing ceratobranchials. In a second approach, we have studied the necessity of VEGF, an important angioneurin, for proper development of the teeth in zebrafish. We have demonstrated that blocking VEGF downstream signalling delays development of replacement teeth in zebrafish, highlighting a role for VEGF during zebrafish odontogenesis.

In conclusion, our research has provided essential baseline information for future studies aimed at unravelling the possible connection between neurovascular networks and tooth development in this model organism.



SUMMARY



5. SUMMARY

5.1 ENGLISH SUMMARY

While there has been an enormous progress of knowledge with regard to the genetic and molecular mechanisms that regulate (mammalian) tooth development (Jernvall and Thesleff, 2000, Thesleff, 2003, Tucker and Sharpe, 2004, Klein et al., 2006, Prochazka et al., 2010), the role of angiogenesis and neurogenesis in this process remain poorly studied. Nonetheless, in order to achieve full development, attachment and maintenance of teeth that can also fulfil their sensory function, proper vascularization and innervation must inevitably accompany their development. The connection between vascular and neural development, maintenance and functioning is termed the 'neurovascular link' and has received renewed interest over the last few years (Carmeliet, 2003b, Carmeliet and Tessier-Lavigne, 2005, Segura et al., 2009, Tam and Watts, 2010). In the present work, we have initiated research on the role of the neurovascular link during tooth development and replacement, taking advantage of a vertebrate animal model that is easily amenable to experimentation, and which is characterized by a lifelong natural replacement of teeth: the zebrafish (*Danio rerio*).

During this PhD, our aim was to test the hypothesis that tooth replacement depends on a properly functioning neurovascular link. However, at the outset, information regarding the innervation and vascularization of zebrafish teeth was completely lacking. Thus, in a descriptive approach, we first studied the vascularization and innervation of the fifth, tooth-bearing, ceratobranchials in zebrafish. In a second, functional, approach, we focussed on vascular endothelial growth factor (VEGF), an angiogenic factor that also appears to be involved in several neurobiological processes (therefore also termed an angioneurin), and studied its role during tooth development and replacement. This was achieved by interfering with its downstream signalling through chemical inhibition.

First, we described the vascular supply to the pharyngeal jaws and teeth in zebrafish using serial high quality semithin sections and 3D reconstructions. We identified that the arterial blood supply to the last pair of branchial arches, which carries the teeth, issues from the hypobranchial artery. Surprisingly, the arteries supplying the pharyngeal jaws showed an asymmetric branching pattern that is modified over ontogeny. Moreover, the blood vessel pattern that serves each jaw can best be described as a sinusoidal cavity encircling the bases of both the functional and replacement teeth. Capillaries branching from this sinusoidal cavity

enter the pulp at a late stage of differentiation, and constitute the intrinsic blood supply to the attached teeth.

Next, we identified the nerves innervating the pharyngeal jaws and teeth using serial semithin section histology and immunohistochemistry. The last pair of branchial arches, which are non-gill bearing, but which carry the teeth, are innervated by an internal branch of a posttrematic ramus of the vagal nerve. Another, external, branch is probably responsible for the motor innervation of the branchiomeric musculature. Nerve fibres, like blood vessels, were found to appear in the pulp cavity of the teeth only late during cytodifferentiation.

Finally, using a functional approach, we examined the role of blood vessels in the dentition of the zebrafish through application of SU5416, a vascular endothelial growth factor receptor inhibitor. We were unable to show an effect on the development of first-generation teeth as well as first tooth replacement. However, in juvenile fish, a delay was observed in the developmental state of the replacement tooth compared with what was expected based on the maturation state of the functional tooth. Furthermore, we observed a difference between treated and non-treated fish in the distance between the nearest blood vessel (i.e., the sinusoidal cavity), and the developing replacement tooth. Nonetheless, new replacement teeth were still initiated. These results provide support for a nutritive, rather than an inductive, function of the vasculature in the process of tooth development and replacement. Tooth initiation might still occur due to the presence of either epithelial or mesenchymal stem cells carried by the vasculature or innervation outside the functional tooth.

The identification of an unusual vascular structure in the dentition of zebrafish, which we termed the sinusoidal cavity, led us to study the dentition of two other taxa with continuous tooth replacement, highly relevant from a phylogenetic perspective: a chondrichthyan (*Scyliorhinus canicula*) and a basal osteichthyan (*Polypterus senegalus*). The presence of a similar sinusoidal cavity not only in two species of ray-finned bony fish, but also in a cartilaginous fish, led us to propose the hypothesis that the sinusoidal cavity might represent an evolutionary adaptation of the vascular system allowing the continuous replacement of teeth. However, given that reptiles do not appear to possess a sinusoidal cavity, its function would rather serve in slowing down the blood stream to secure an efficient exchange of oxygen and nutrients. Alternatively, the sinusoidal cavity might be connected to the single circulatory system present in fish.

Based on morphological data, it is difficult to infer the presence of a neurovascular link. Superficially, both vascular and neural networks do not appear to line up for wiring of the dentition in zebrafish, at least not at a gross anatomical level. However, alignment of blood

vessels and nerves might still occur at each individual tooth position between smaller branches of both vessels and nerves running towards the pulp. This could be achieved through the secretion of growth factors by developing tooth germs, vessels, or nerves.

In conclusion, our research has provided the scientific community with essential baseline information, and has set the stage for future studies aimed at unravelling the possible connection between neurovascular networks and tooth development in the zebrafish.

5.2 NEDERLANDSE SAMENVATTING

Ondanks de enorme vooruitgang die de laatste jaren gemaakt werd op gebied van de genetische en moleculaire mechanismen die instaan voor (zoogdier)tandontwikkeling (Jernvall and Thesleff, 2000, Thesleff, 2003, Tucker and Sharpe, 2004, Klein et al., 2006, Prochazka et al., 2010), zijn bepaalde aspecten nog nauwelijks of niet bestudeerd. De rol van angiogenese en neurogenese in dit proces is daar een voorbeeld van. Echter, voor een goede ontwikkeling, vasthechting en onderhoud van tanden, die ook hun sensorische functie kunnen uitoefenen, is een correcte bloedvoorziening en bezenuwing tijdens hun aanleg essentieel. De overeenkomsten tussen het bloedvatenstelsel enerzijds en het zenuwstelsel anderzijds, noemt men de 'neurovasculaire link' en heeft de afgelopen jaren heel wat hernieuwde interesse verworven (Carmeliet, 2003b, Carmeliet and Tessier-Lavigne, 2005, Segura et al., 2009, Tam and Watts, 2010). Voor deze studie hebben we gebruik gemaakt van een modelorganisme dat zich makkelijk leent voor experimenteel onderzoek, en gekenmerkt wordt door een levenslange natuurlijke tandvervanging: de zebravis (*Danio rerio*).

Gedurende dit doctoraat was het ons doel om de hypothese te testen dat tandvervanging afhangt van een correct functionerende neurovasculaire link. Echter, bij de aanvang van deze studie was er geen informatie beschikbaar betreffende de innervatie en vascularisatie van zebravistanden. Dus, in een beschrijvende methode werd enerzijds de innervatie en anderzijds de vascularisatie van de vijfde tanddragende ceratobranchialen in zebravis bestudeerd. In een tweede functionele benadering werd gedurende tandontwikkeling –en vervanging de rol onderzocht van de vasculaire endotheliale groeifactor (VEGF), een angiogene factor die betrokken is bij tal van neurobiologische processen (daarom ook wel een angioneurine genaamd). Hierbij werd de signalisatie pathway geblokkeerd via de toediening van een inhibitor.

Vooreerst werd de bloedtoevoer naar de faryngeale kaken en tanden beschreven in zebravis gebruik makend van seriële semidunne coupes en 3D reconstructies. We hebben de hypobranchiale arterie geïdentificeerd als zijnde verantwoordelijk voor de bloedtoevoer naar het laatste paar tanddragende branchiale bogen. Verrassend genoeg vertoonden de arteriën die de faryngeale kaken voorzien van bloed een asymmetrisch vertakkingspatroon dat gemodificeerd wordt gedurende de ontwikkeling. Bovendien, kon het bloedvaten patroon ter hoogte van de tanden best beschreven worden als een sinusoïdale holte die de basis van zowel functionele als vervangingstanden omsluit. Kleinere capillairen die aftakken van deze holte

penetreren in de pulpaholte gedurende late cytodifferentiatie en verzorgen de intrinsieke bloedtoevoer van de aangehechte tanden.

Vervolgens hebben we de zenuwen geïdentificeerd die de faryngeale kaken innerveren door middel van histologie en immunohistochemische kleuringen. Het laatste paar van branchiale bogen, welke niet kieuw- maar tanddragend zijn, worden geïnnerveerd door een interne tak van de posttrematische ramus van de tiende craniale zenuw, de vagus. Een andere externe tak is wellicht verantwoordelijk voor de motorische innervatie van de branchiale musculatuur. Zenuwvezels, net zoals bloedvaten, zijn pas gedetecteerd in de pulpaholte vanaf late cytodifferentiatie.

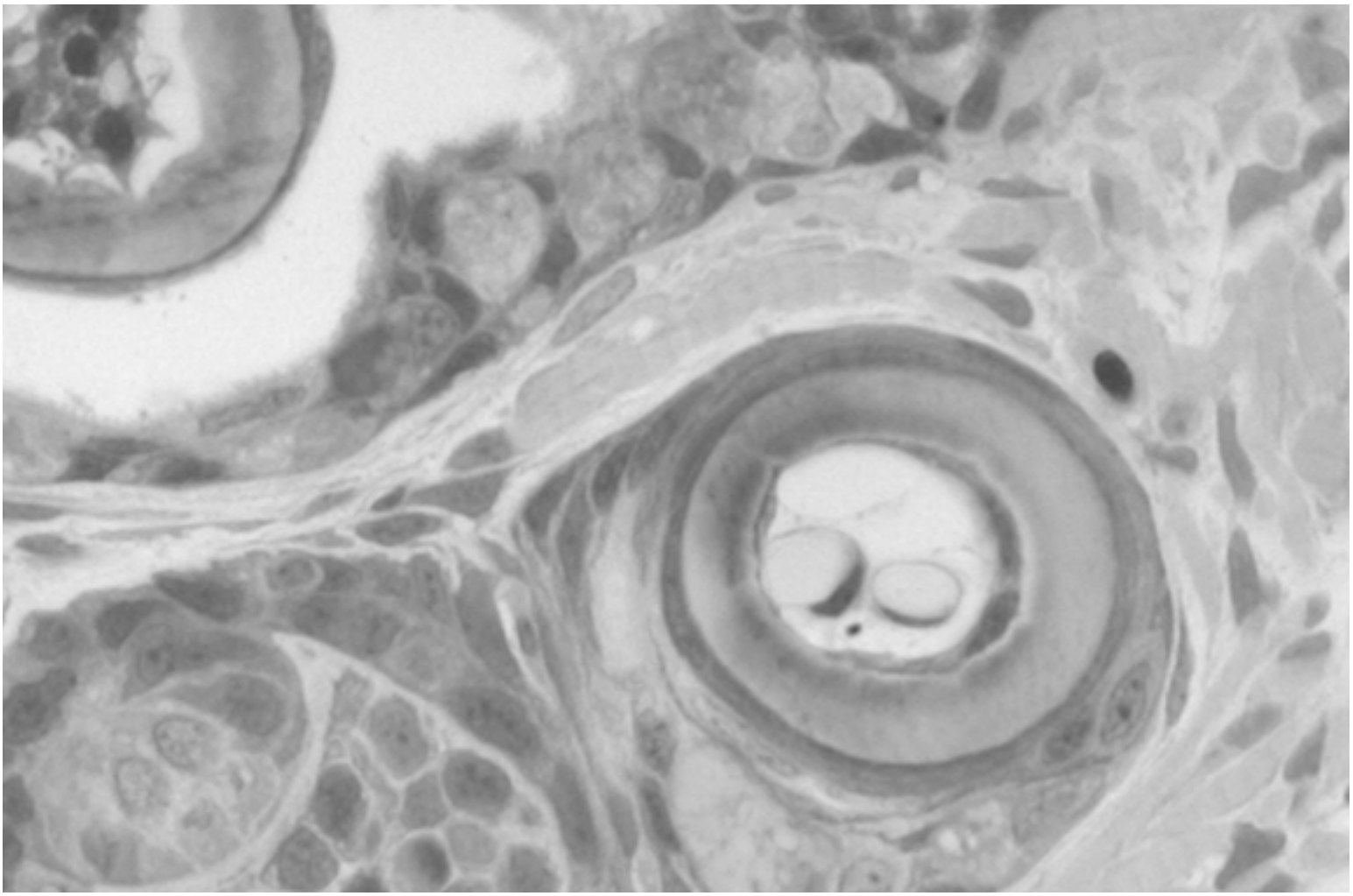
Tot slot hebben we via een functionele aanpak de rol bestudeerd van de bloedvaten in het gebit van zebravis door gebruik te maken van SU5416, een VEGF receptor inhibitor. Voor zowel de ontwikkeling van eerste generatie tanden als voor de eerste tandvervanging waren we niet in staat om een effect aan te tonen. Desondanks zijn we er wel in geslaagd om in juveniele vissen een vertraging vast te stellen op basis van de ontwikkelingstoestand van de vervangingstand ten opzichte van de graad van maturatie van de corresponderende functionele tand. Bovendien hebben we een verschil aangetoond in de afstand van de bloedvaten tot de ontwikkelende vervangingstanden tussen behandelde en niet behandelde dieren. Niettegenstaande werden nieuwe vervangingstanden toch nog geïnitieerd. Resultaten van deze aanpak duiden eerder op een nutritieve dan inductieve functie van de bloedvaten gedurende het proces van tandontwikkeling en vervanging in zebravis. Tandinitiatie kan echter wel nog tot stand komen door de aanwezigheid van epitheliale of mesenchymale stamcellen gedragen door de bloedvaten of zenuwen buiten de functionele tand.

Gedurende dit doctoraat hebben we de eerste stappen ondernomen om de rol te bestuderen van de neurovasculaire link gedurende natuurlijke tandvervanging in zebravis. Tijdens dit proces hebben we een grote vasculaire structuur geïdentificeerd in het gebit van zebravis welke we de sinusoïdale holte genoemd hebben. De ontdekking van deze unieke structuur heeft aanleiding gegeven tot de studie van de vascularisatie in het gebit van twee andere fylogenetisch relevante soorten met continue tandvervanging: een kraakbeenvis (*Scyliorhinus canicula*) en een basale beenvis (*Polypterus senegalus*). Omwille van het feit dat we niet alleen in twee soorten straalvinnige beenvissen maar ook in een kraakbeenvis een sinusoïdale holte hebben kunnen identificeren, hebben we de hypothese naar voor geschoven dat de sinusoïdale holte een mogelijke adaptatie is van het bloedvatenstelsel om de continue vervanging van tanden in stand te houden. Daarentegen bezitten reptielen wellicht geen sinusoïdale holte. Hierdoor zou de functie van de sinusoïdale holte eerder zijn om de

bloedstroom te vertragen voor een efficientie uitwisseling van bouwstenen en zuurstof. Anderzijds zou de sinusoidale holte het gevolg kunnen zijn van het enkelvoudig circulatorisch systeem in vissen.

Het is moeilijk om op basis van onze morfologische data de aanwezigheid van een neurovasculaire link af te leiden. Oppervlakkig gezien lijken zowel het vasculair als het neuraal stelsel zich niet met elkaar te aligneren gedurende de tandontwikkeling bij zebravis. Nochtans kan het samenlopen van zenuwen en bloedvaten toch nog optreden bij elke individuele tandpositie tussen kleinere vertakkingen van zowel het bloedvaten- als zenuwstelsel die naar de pulpaholte gaan. Dit zou kunnen gebeuren door de secretie van groeifactoren door ontwikkelende tanden, bloedvaten of zenuwen.

Als conclusie kunnen we stellen dat ons onderzoek de nodige essentiële basisinformatie heeft aangeleverd voor toekomstige studies die de connectie tussen neurovasculaire netwerken en tandontwikkeling in zebravis willen bestuderen.



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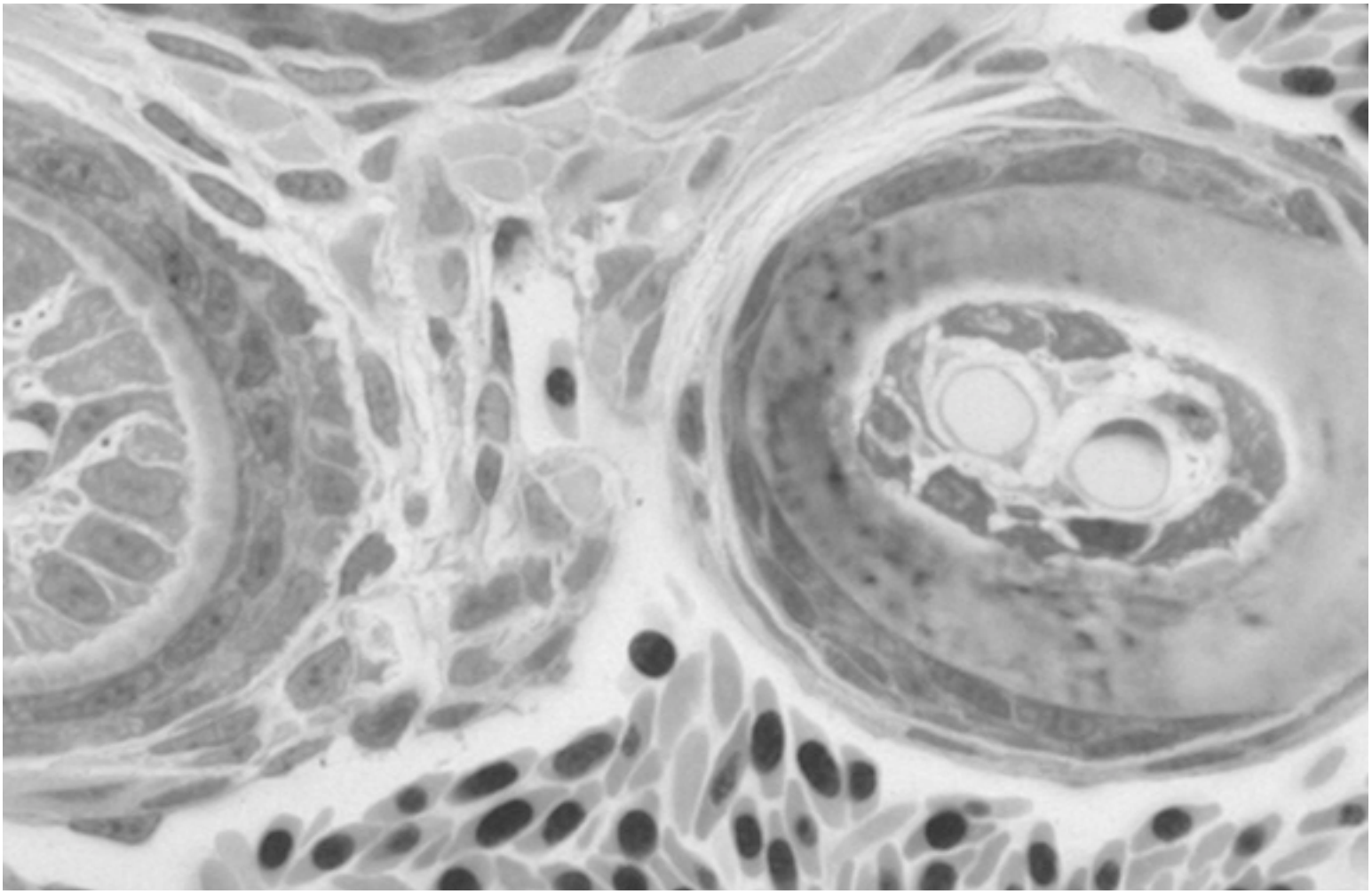

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